

TRANSCRIPT LEVEL	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
Down-regulated transcripts (level at 1 hour < 6 hours)	"Delayed" repressor responders to Auxin  Genes for pathways diminished in presence of Auxin	<ul style="list-style-type: none"> <li>• Maintenance of Auxin stimulated state(s) in certain cells</li> <li>• Reorientation of metabolism in certain cells</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in activity of cytoskeletal proteins modulating cell structure</li> <li>• Changes in chromatin structure and/or DNA topology</li> <li>• Changes in protein structure and/or function by phosphorylation (kinases) and/or dephosphorylation (phosphatases)</li> <li>• Stability of factors for protein translation</li> <li>• Changes in cell membrane structure</li> <li>• Changes in chromatin and/or localized DNA topology</li> <li>• Changes in protein-protein interaction</li> <li>• Metabolic enzymes</li> </ul>

#### USE OF PROMOTERS OF NAA RESPONSIVE GENES

Promoters of NAA responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the NAA responsive genes where the desired sequence is operably linked to a promoter of a NAA responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### III.C.3. BRASSINOSTEROID RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS:

Plant hormones are naturally occurring substances, effective in very small amounts, which act as signals to stimulate or inhibit growth or regulate developmental processes in plants.

Brassinosteroids (BRs) are the most recently discovered, and least studied, class of plant hormones. The major physiological response affected by BRs is the longitudinal growth of young tissue via cell elongation and possibly cell division. Consequently, disruptions in BR metabolism, perception and activity frequently result in a dwarf phenotype. In addition, because BRs are derived from the sterol metabolic pathway, any perturbations to the sterol pathway can affect the BR pathway. In the same way, perturbations in the BR pathway can have effects on the later part of the sterol pathway and thus the sterol composition of membranes.

Changes in BR concentration in the surrounding environment or in contact with a plant result in modulation of many genes and gene products. Examples of such BR responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant biomass and seed yield. These genes were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA abundance changed in response to application of BRs to plants.

While BR responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different BR responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factors and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a BR responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways. The

MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108580, 108581, 108557, 108478, 108479, 108480, 108481). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

BR genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### BR Genes Identified By Cluster Analyses Of Differential Expression

##### BR Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of BR genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108580, 108581, 108557, 108478, 108479, 108480, 108481 of the MA\_diff table(s).

##### BR Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of BR genes. A group in the MA\_clust is considered a BR pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

##### BR Genes Identified By Amino Acid Sequence Similarity

BR genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis BR genes. Groups of BR genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID

that corresponds to a cDNA ID member of a BR pathway or network is a group of proteins that also exhibits BR functions/utilities.

Such BR responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in BR concentration or in the absence of BR fluctuations. Further, promoters of BR responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by BR or any of the following phenotypes or biological activities below.

III.C.3.a. Use Of Brassinosteroid Responsive Genes To Modulate Phenotypes

Brassinosteroid responsive genes and gene products are useful to modulate one or more phenotypes including growth (promotes cell elongation, elongation accelerated at low temperatures for increased plant growth in marginal lands, acts in concert with other hormones to promote cell division); roots (inhibitory to root growth, and expression in roots would inhibit bud breaking due to higher auxin:cytokinin ratio in epicotyl); stems (inhibits radial growth while causing stem elongation, in low concentrations, promotes radial expansion, and increases biomass); height; seeds; promotes cell expansion in embryo and thus enhances germination; leaves; increase biomass; flowers, increase reproduction; biomass; fresh and dry weight during any time in plant life, such as maturation; number of flowers; number of seeds; number of branches; number of leaves; starch content; seed yield (including number, size, weight, harvest index, starch content; fruit yield, number, size, weight, harvest index, and starch content); development; morphogenesis; control of organ size and shape; development of new ornamentals; control of leaf size and shape; promotes leaf unrolling and enlargement; for development of new leafy ornamentals; seed development; inhibition of de-etiolation; dormancy; accelerated germination at low temperatures; root; gravitropism; senescence; promoted in light grown plants; inhibiting synthesis or perception could extend life span of desired tissues/organs; differentiation; vascularization; promotes xylem differentiation; increases xylem fiber length ; resistance responses; increases resistance to pathogens; and tropic responses.

Gravitropic Responses Affecting Roots

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the BR responsive genes when the desired sequence is operably linked to a promoter of a BR responsive gene.

To improve any of the desired phenotype(s) above, activities of one or more of the BR response genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266, and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50, visually inspected for the desired phenotype and metabolically and/or functionally assayed according to Choe et al. (1999, Plant Cell 11:207-21 and Plant Physiol 119: 897-907), Yamamoto et al. (1997, Plant Cell Physiol 38:980-3), Asami and Yshida (1999, Trends in Plant Sciences, 4:348-353) and Azpiroz et al. (1998, Plant Cell 10:219-230)

III.C.3.b. Use Of Brassinosteroid Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the BR responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
BR Transport	<ul style="list-style-type: none"><li>• BR Efflux Between Cells</li></ul>	B.Schulz and K. Feldmann, unpub. results
	<ul style="list-style-type: none"><li>• BR Influx In And Out Of A Cell</li></ul>	B.Schulz and K. Feldmann, unpub. results
Signal Transduction	<ul style="list-style-type: none"><li>• Permeability Of Cell Membranes</li></ul>	
	<ul style="list-style-type: none"><li>• Protein Phosphorylation</li></ul>	
Metabolism	<ul style="list-style-type: none"><li>• Major Growth Coordinating</li></ul>	

	Pathways	
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Other biological activities that can be modulated by the BR responsive genes and gene products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Domain section of the Reference Tables.

BR responsive genes are differentially transcribed in response to fluctuating BR levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various BR responsive genes in the aerial parts of a seedling at 1 and 6 hours after a plant was sprayed with a solution enriched with BR as compared to seedlings sprayed with water. The data from this time course can be used to identify a number of types of BR responsive genes and gene products, including "early responders," "delayed responders." Profiles of these different categories of BR responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up Regulated Transcripts (Level At 1 Hr ≈ 6 Hr) (Level At 1 Hr > 6 Hr)	• Early Responders To BR	<ul style="list-style-type: none"> <li>• BR Perception</li> <li>• BR Transport</li> <li>• BR Biosynthesis Feedback</li> <li>• Modulation Of BR Response Transduction Pathways</li> <li>• Specific Gene Transcription Initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Receptors</li> <li>• Transporters</li> <li>• Change In Cell Membrane Structure</li> <li>• Feedback Regulated Biosynthetic Genes</li> <li>• Kinases And Phosphatases</li> <li>• 2<sup>nd</sup> Messengers, Eg., Calmodulin</li> <li>• Transcription Activators</li> <li>• Change In Chromatin Structure And/Or Localized DNA Topology</li> </ul>
Up Regulated Transcripts (Level At 1 Hr < 6	• Delayed Responders	• Maintenance Of Response To Br	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• BR Biosynthetic</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Hr)			<ul style="list-style-type: none"> <li>• Genes</li> <li>• Specific Factors (Initiation And Elongation) For Protein Synthesis</li> <li>• Maintenance Of Mrna Stability</li> <li>• Maintenance Of Protein Stability</li> <li>• Maintenance Of Protein-Protein Interaction</li> <li>• Cell Wall Elongation</li> <li>• Cell And Organ Elongation</li> <li>• Gravitropism</li> </ul>
Down-Regulated Transcripts (Level At 1 Hr ≈ 6 Hr) (Level At 6 Hr > 1 Hr)	Early Responder Repressors Of BR State Of Metabolism  Genes With Discontinued Expression Or Unstable Mrna In Presence Of BR	<ul style="list-style-type: none"> <li>• Negative Regulation Of BR Pathways Released</li> <li>• Changes In Pathways And Processes Operating In Cells</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Change In Protein Structure By Phosphorylation (Kinases) Or Dephosphoryaltion (Phosphatases)</li> <li>• Change In Chromatin Structure And/Or DNA</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
			Topology
Down-Regulated Transcripts (Level At 1 Hr > 6 Hr)	<ul style="list-style-type: none"> <li>• Delayed Repressors Of BR State Of Metabolism</li> <li>• Genes With Discontinued Expression Or Unstable Mrna In Presence Of BR</li> </ul>	<ul style="list-style-type: none"> <li>• Negative Regulation Of BR Pathways Released</li> <li>• Maintenance Of Pathways Released From Repression</li> <li>• Changes In Pathways And Processes Operating In Cells</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Kinases And Phosphatases</li> <li>• Stability Of Factors For Protein Synthesis And Degradation</li> </ul>

#### USE OF PROMOTERS OF BR RESPONSIVE GENES

Promoters of BR responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the BR responsive genes where the desired sequence is operably linked to a promoter of a BR responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### III.C.4. CYTOKININ RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Plant hormones are naturally occurring substances, effective in very small amounts, which act as signals to stimulate or inhibit growth or regulate developmental processes in plants. Cytokinins (BA) are a group of hormones that are best known for their stimulatory effect on cell division, although they also participate in many other processes and pathways. All naturally occurring BAs are aminopurine derivatives, while nearly all synthetic compounds with BA activity are 6-substituted aminopurine derivatives. One of the most common synthetic BAs used in agriculture is benzylaminopurine (BAP).

Changes in BA concentration in the surrounding environment or in contact with a plant results in modulation of many genes and gene products. Examples of such BA responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff and MA\_clust. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to application of BA to plants.

While cytokinin responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different BA responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a BA responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping

pathways. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108566, 108567, 108517). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

BA genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### BA Genes Identified By Cluster Analyses Of Differential Expression

##### BA Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of BA genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108566, 108567, 108517 of the MA\_diff table(s).

##### BA Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of BA genes. A group in the MA\_clust is considered a BA pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

##### BA Genes Identified By Amino Acid Sequence Similarity

BA genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis BA genes. Groups of BA genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a BA pathway or network is a group of proteins that also exhibits BA functions/utilities.

Such BA responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in BA concentration or in the absence of BA fluctuations.

Further, promoters of BA responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by BA or any of the following phenotypes or biological activities below.

#### III.C.4.a. Use Of Ba-Responsive Genes To Modulate Phenotypes

BA responsive genes and gene products are useful to or modulate one or more phenotypes including growth, roots (such as inhibition of elongation of root); stems (such as inhibition of elongation of hypocotyl); lateral buds (such as promotion of outgrowth for rapid production of multiple shoots as a source for grafting); leaves such as development (including cell growth, such as expansion of cotyledon and promotes cell enlargement for increased yield from leaf crops, chloroplast development such as delayed degradation of chloroplasts for increased photosynthesis and crop yield, cell division and senescence such as delays for delayed conversion from photosynthesis to salvage programs in leaves and for increased crop yield); differentiation such as regulation of morphogenesis for manipulating callus growth and shoot/root formation in culture; maintenance of shoot meristem such as for increased usable wood production, and reduced tiller number for denser crop planting regimes; nutrient metabolism for effects on seed size and effects on rate of seed set for increased seed yield; induction of ethylene biosynthesis for control of fruit ripening; and parthenocarpy for control of sexual reproduction and production of seedless fruits.

To regulate any of the phenotype(s) above, activities of one or more of the BA responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or molecularly or metabolically or functionally assayed according to Lohman et al (1994, Physiol. Plant 92:322-328), Woolhouse (1983, In Agricultural Research-

Strategies of Plant reproduction, Meudt, ed., 201-236), Medford et al. (1989, Plant Cell 1: 403-13), Vogel et al. (1998, Genetics 149:417-27), Ehnes and Roitsch (1997, Plant J 1: 539-48), Rotino et al. (1997, Nat. Biotechnol. 15: 1398-1401).

**III.C.4.b. Use Of Ba-Responsive Genes To Modulate Biochemical Activities**

The activities of one or more of the BA responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Chloroplast Functioning	• Photosynthesis	Běnková et al (1999) Plant Physiol 121: 245-252
Induction And Maintenance Of Cell Division	• Cell Cycle Phase Transition	Riou-Khamlich et al. (1999) Science 283: 1541-44
Senescence	• Cell Death/Apoptosis	Lohman et al. (1994) Physiol Plant 92: 322-328
Signal Transduction	• Sensing Endogenous Stimuli To Trigger Growth And Shoot Formation	Kakimoto (1996) Science 274: 982-985

Other biological activities that can be modulated by the BA responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Domain section above.

BA responsive genes are characteristically differentially transcribed in response to fluctuating BA levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various BA responsive genes in the

aerial parts of a seedling at 1 and 6 hours after a plant was sprayed with a Hoagland's solution enriched with BA as compared to seedlings sprayed with Hoagland's solution only.

The data from this time course can be used to identify a number of types of BA responsive genes and gene products, including "early responders," and "delayed responders." Profiles of these different BA responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENES	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up Regulated Transcripts (Level At 1h $\geq$ 6h) Or (Higher At 1h Than 6h)	• Early Responders To BA	- BA Perception -BA Uptake -Modulation Of BA Response Transduction Pathways -Specific Gene Transcription Initiation -Initiate And Coordinate Cell Division	-Transcription Factors -Transporters -Kinase, Receptor-Like Protein Kinase  -Ovule-Specific Homeotic Protein, Secretory Pathway -Cell Division Control Protein, Cyclins, Cyclin-Dependent Protein Kinase (Cdk), Cell Cycle Phosphatases, Mitosis-Specific Chromosome Segregation Protein, Mitotic Phosphoprotein, Dna Replication Proteins, Hélicase Telomerase, Centromere Protein,

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENES	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
		-Regulation Of Pathways To Senescence	tRNA Synthase -Senescence-Associated Protein, Bifunctional Nuclease, Aba Pathway Genes, Ethylene Pathway Genes, Proteases, Nucleases, Pcd Genes -Calvin Cycle, Chlorophyll A/B Binding Protein (Cab), Transketolase, Lipoxygenase, Chloroplast Rna Processing Protein, Chloroplast Envelope Membrane Protein. -Glutamate Synthase, Gogat, Asparagine Synthase, Catalase, Peroxidase
		-Modulation Of Chloroplast Gene Expression And Photosynthesis	-Heat Shock Proteins, Gst -Fatty Acid Elongase-Like Protein, Very-Long-Chain Fatty Acid Condensing Enzyme, Coa
		-Modulation Of Photorespiration And Primary Nitrogen Assimilation In Leaves	

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENES	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>-Expression</li> <li>-Stress Response</li> <li>-Wax Biosynthesis</li> <li>-Nutrient Metabolism</li> <li>-Embryogenesis</li> <li>-Glycolysis,</li> <li>Gluconeogenesis</li> <li>-Ripening</li> </ul>	<ul style="list-style-type: none"> <li>Synthase</li> <li>-Vicilin Storage Protein</li> <li>-Homeobox Domain Proteins</li> <li>-Mutase, Phosphoglycerate Mutase</li> <li>-Pectate Lyase, Ethylene Pathway Genes</li> </ul>
Upregulated Transcripts (Higher At 6h Than 1h)	BA Late Responders	<ul style="list-style-type: none"> <li>-BA Responsive Pathways</li> <li>-Cell Wall Extension</li> <li>-Organogenesis</li> <li>-Modulate Activation</li> </ul>	<ul style="list-style-type: none"> <li>-Transfactors, Kinases, Phosphatases, LRR's, Dna Remodelling Proteins, Cu-Binding Proteins</li> <li>-Expansins, Extensins, Proline Rich Proteins</li> <li>-AP2 Domain Containing Proteins</li> <li>-Transfactors Interacting</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENES	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>-Of Disease Defense Genes</li> <li>-Modulate Responses To External Stimuli</li> <li>-Osmotic Stress Tolerance</li> </ul>	<ul style="list-style-type: none"> <li>-With Resistant Genes</li> <li>-Glycin-Rich Proteins, Wall-Associated Receptor Kinase (Wak)</li> <li>-Proline Oxidase</li> </ul>
Down-Regulated Transcripts (Low At Both 1h and 6h)	Repressors Of BA Pathway	<ul style="list-style-type: none"> <li>-Regulation Of Senescence-Related Gene Expression</li> <li>-Regulation Of Genes Involved In Maintenance Of Apical Dominance.</li> </ul>	<ul style="list-style-type: none"> <li>-Transfactors (Such As Zinc-Finger Type), Kinases, Phosphatases, G-Proteins, LRR Proteins, DNA Remodeling Protein Carbonyl Reductases</li> <li>-Atpases</li> <li>-Oxygenase</li> <li>-Octaprenyltransferase</li> <li>-Auxin Pathway Genes</li> <li>-Auxin Binding Proteins</li> </ul>

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the BA responsive genes when the desired sequence is operably linked to a promoter of a BA responsive gene.

### III.C.5. GIBBERELLIC ACID RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Plant hormones are naturally occurring substances, effective in very small amounts, which act as signals to stimulate or inhibit growth or regulate developmental processes in plants.

Gibberellic acid (GA) is a hormone in vascular plants that is synthesized in proplastids (giving rise to chloroplasts or leucoplasts) and vascular tissues. The major physiological responses affected by GA are seed germination, stem elongation, flower induction, anther development and seed and pericarp growth. GA is similar to Auxins, cytokinins and gibberellins, in that they are principally growth promoters.

Changes in GA concentration in the surrounding environment or in contact with a plant result in modulation of many genes and gene products. Examples of such GA responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and biomass and seed yield. They were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA products changed in concentration in response to application of nitrogen to plants.

While GA responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different GA responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways and/or segments of pathways are controlled by transcription factors and proteins that affect the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a GA responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common overlapping pathways. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108562, 108563, 108519,

108520, 108521, 108484, 108485, 108486). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

GA genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

GA Genes Identified By Cluster Analyses Of Differential Expression

GA Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of GA genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108562, 108563, 108519, 108520, 108521, 108484, 108485, 108486 of the MA\_diff table(s).

GA Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of GA genes. A group in the MA\_clust is considered a GA pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

GA Genes Identified By Amino Acid Sequence Similarity

GA genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis GA genes. Groups of GA genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a GA pathway or network is a group of proteins that also exhibits GA functions/utilities.

Such GA responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in GA concentration or in the absence of GA fluctuations. Further, promoters of GA responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by GA or any of the following phenotypes or biological activities below.

**III.C.5.a. Use Of GA Responsive Genes To Modulate Phenotypes:**

GA responsive genes and gene products are useful to or modulate one or more phenotypes including growth, promotes root growth, promotes cell division, promotes stem elongation, secondary (woody) growth, promotes growth in leaves, biomass, increase in stem and leaf mass, increase in xylem fiber length and biomass production, development, cell growth, fruit development, seed development, dormancy, breaks dormancy in seeds and buds, promotes trichome formation, decrease senescence, regulation of fertility, stress responses, and flowering time.

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the GA responsive genes when the desired sequence is operably linked to a promoter of a GA responsive gene.

To regulate any of the phenotype(s) above, activities of one or more of the GA response genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Hedden and Proebsting (1999, Plant Physiol. 119:365-370), Hedden and Phillips (1999, Current Opinion in Plant Biotech. 11:130-137), Perazza et al (1998, Plant Physiol. 117:375-383), Kende and Zeevat (1997, Plant Cell 9:1197-1210) and van der Knaap et al. (2000, Plant Physiol. 122:695-704).

**III.C.5.b. Use Of GA-Responsive Genes To Modulate Biochemical**

Activities:

The activities of one or more of the GA responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cell Growth and Differentiation	Biosynthesis of Gas  Cell wall loosening and cell expansion  GA deactivation  Major growth promoting metabolic pathways	Hedden and Proebsting (1999, Plant Physiol. 119:365-370)  Cosgrove (1993, New Phytol. 124:1-23)  Hedden and Proebsting (1999, Plant Physiol. 119:365-370)
Perception and Signal Transduction	Receptors	Koornneef and van der Veen (1980, TAG 58:257-263)
	Synthesis of transcriptional regulators  Calcium and Calmodulin	Bethke and Jones (1998, Curr. Opin. Plant Biol. 1:440-446)

Other biological activities that can be modulated by the GA responsive genes and gene products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Protein Domain table.

GA responsive genes are characteristically differentially transcribed in response to fluctuating GA levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various GA responsive genes in

entire seedlings at 1 and 6 hours after a plant was sprayed with a Hoagland's solution enriched with GA as compared to seedlings sprayed with Hoagland's solution only.

The data from this time course can be used to identify a number of types of GA responsive genes and gene products, including "early responders," and "delayed responders." Profiles of some GA responsive genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts  (level at 1 hr ≈ 6 hr)	Early responders to GA  Genes induced by GA	GA perception  GA transport  Modulation of GA response  transduction pathways  Specific gene transcription initiation  Growth stimulating pathway induction	Transcription factors  Transporters  Change in cell membrane structure  Kinases and phosphatases  Transcription activators  Change in chromatin structure and/or localized DNA topology  Cell wall proteins  Metabolic Enzymes
Up regulated transcripts  (level at 1 hr < 6 hr)	Maintenance of GA response  "Delayed" responders	Maintenance of response to GA  Induction of GA metabolic pathways	Transcription factors  Specific factors  (initiation and elongation) for protein synthesis  Maintenance of mRNA

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
			stability Metabolic enzymes
Down-regulated transcripts  (level at 1 hr ≈ 6 hr)	Early repressor responders to GA	Negative regulation of GA pathways released	Transcription factors Calmodulin Change in protein structure by phosphorylation (kinases) or dephosphorylation (phosphatases)
(level at 6 hr > 1 hr)	Genes repressed by GA  Genes whose activities are diminished or mRNAs are unstable in the presence of GA	Reduced activity of repressed pathways	Change in chromatin structure and/or DNA topology
Down-regulated transcripts  (level at 1 hr > 6 hr)	Delayed responders  Genes repressed by GA  Genes whose activities are diminished or mRNAs are unstable in the presence of GA	Maintenance of GA repressed pathways	Transcription factors Kinases and phosphatases Stability factors for protein translation Metabolic enzymes

#### USE OF PROMOTERS OF GA RESPONSIVE GENES

Promoters of GA responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in

a similar temporal, tissue, or environmentally specific patterns as the GA responsive genes where the desired sequence is operably linked to a promoter of a GA responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.D. METABOLISM AFFECTING GENES, GENE COMPONENTS AND PRODUCTS**

#### **III.D.1. NITROGEN RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Nitrogen is often the rate-limiting element in plant growth, and all field crops have a fundamental dependence on exogenous nitrogen sources. Nitrogenous fertilizer which is usually supplied as ammonium nitrate, potassium nitrate, or urea, typically accounts for 40% of the costs associated with crops, such as corn and wheat in intensive agriculture. Increased efficiency of nitrogen use by plants should enable the production of higher yields with existing fertilizer inputs and/or enable existing yields of crops to be obtained with lower fertilizer input, or better yields on soils of poorer quality. Also, higher amounts of proteins in the crops could also be produced more cost-effectively.

Changes in nitrogen concentration in the surrounding environment or in contact with a plant results in modulation of the activities of many genes and hence levels of gene products. Examples of such “nitrogen responsive” genes and gene products with these properties are shown in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff, MA\_clust, Knock-in and Knock-out tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to changing levels of available nitrogen to plants.

Manipulation of one or more “nitrogen responsive” gene activities is useful to modulate the biological activities and/or phenotypes listed below. “Nitrogen responsive” genes and gene products can act alone or in combination. Useful combinations include nitrogen responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of the levels of such proteins is especially useful for altering phenotypes and biochemical activities of plants. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108592, 108593, 108588,

108589, 108590, 108591, 108532, 108548, 108549, 108550, 108551, 108454, 108455, 108487, 108488, 108489, and Nitrogen (relating to SMD 3787, SMD 3789)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Nitrogen genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Nitrogen Genes Identified By Cluster Analyses Of Differential Expression

#### Nitrogen Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Nitrogen genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108592, 108593, 108588, 108589, 108590, 108591, 108532, 108548, 108549, 108550, 108551, 108454, 108455, 108487, 108488, 108489, and Nitrogen (relating to SMD 3787, SMD 3789) of the MA\_diff table(s).

#### Nitrogen Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Nitrogen genes. A group in the MA\_clust is considered a Nitrogen pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

#### Nitrogen Genes Identified By Amino Acid Sequence Similarity

Nitrogen genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and *Arabidopsis* Nitrogen genes. Groups of Nitrogen genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Nitrogen pathway or network is a group of proteins that also exhibits Nitrogen functions/utilities.

Such "nitrogen responsive" genes and gene products can function either to either increase or dampen the phenotypes and activities below, either in response to changes in nitrogen concentration or in the absence of nitrogen fluctuations.

Further, promoters of nitrogen responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by nitrogen or any of the following phenotypes or biological activities below.

III.D.5.a. Use Of Nitrogen-Responsive Genes To Modulate Phenotypes

"Nitrogen responsive" genes and gene products can be used to alter or modulate one or more phenotypes including plant development, initiation of the reproduction cycle from a vegetative state (such as flower development time and time to fruit maturity); root development and initiation (such as root branching, lateral root, initiation and/or development, nodule formation and nitrogen assimilation from any nitrogen-fixing symbions), growth rate, whole plant (including height, flowering time, etc.), organs (such as flowers, fruits, stems, leaves, roots, and lateral roots), biomass (such as fresh and dry weight during any time in plant life, such as maturation); number, size, and weight of flowers; seeds; branches, and leaves); total plant nitrogen content, amino acid/protein content of whole plant or parts, seed yield (such as number, size, weight, harvest index and content and composition, e.g., amino acid, nitrogen, oil, protein, and carbohydrate) and fruit yield (such as number, size, weight, harvest index, content and composition, e.g., amino acid, nitrogen, oil, protein, carbohydrate, and water).

To regulate any of the phenotype(s) above, activities of one or more of the nitrogen responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in

Winkler et al. (1998) Plant Physiol 118: 743-50 and assayed, for example, in accordance to Zhang (1999) Proc. Natl. Acad. Sci. 96(11): 6529-34; or Zhang and Forde (1998) Science 279(5349):407-9; Scheible, W., Lauerer, M., Schultze, E.-D., Caboche, M., and Sitt, M. (1997). Plant J. 11, 671-691; Chevalier C, Bourgeois E, Just D, Raymond P. Plant J. 1996 Jan;9(1):1-11.

**III.D.5.b. Use Of Nitrogen-Responsive Genes To Modulate Biochemical Activities**

The activities of one or more of the nitrogen responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations included in the Table below:

Process	Biochemical Or Metabolic Activities And/Or Pathways	Citations including assays
Nitrate And Ammonium Uptake and Assimilation	NO <sub>3</sub> <sup>-</sup> Influx And Efflux	Lejay et al. (1999) Plant J. 18(5): 509-519
	Nitrate Channels	Liu et al. (1999) Plant Cell 11: 865-874; and Wang et al.(1998) Proc. Natl. Acad. Sci. USA 95: 15134-15139
	Changes In Membrane-Potential	Meharg et al. (1995) J. Membr. Biol. 145: 49-66; and Wang et al. (1998), supra
Amino Acid Synthesis	Glutamine Synthesis And Then Biosynthesis Of Other Amino Acids	Coruzzi et al. U.S. Pat. No. 5,955,651; and Oliveira et al. (1999) Plant. Phys. 121: 301-309

Process	Biochemical Or Metabolic Activities And/Or Pathways	Citations including assays
	Asparagine Synthesis And Then Biosynthesis Of Other Amino Acids	LAM ET AL. (1998) PLANT J. 16(3): 345-353
Coordination Of Carbon And Nitrogen Metabolism	Light-Regulation Of Major Central Carbon And Nitrogen Metabolic Pathways To Coordinate Growth	Lam et al. (1998), <i>supra</i> ; Lejay et al. (1999), <i>supra</i> ; and Oliveira et al. (1999), <i>supra</i>
	Carbohydrate And Nitrogen Control Of Carbohydrate And Organic Nitrogen Accumulation Pathways	Lam et al. (1998) <i>supra</i> ; Lejay et al. (1999) <i>supra</i> ; and Oliveira et al. (1999) <i>supra</i>
Nitrogen Loading And Unloading	Nitrogen Transport From Source To Sinks	Walker et al. (1999) 210(1):9-18 Elsheikh et al. (1997) 51(2):137-44.
Nitrogen Storage	Accumulation Of Amino Acids And/Or Storage Proteins In Vacuoles	Johnson et al. (1990) Plant Cell 2(6):525-32.  Herman and Larkins (1999) Plant Cell. 11(4):601-14.
Ammonium Detoxification	Plastid Ammonium Storage/Glutamine Synthesis	Crawford (1995) Plant Cell 7(7):859-68.  Zhang and Forde (1998) Science 279: 407-409.

Process	Biochemical Or Metabolic Activities And/Or Pathways	Citations including assays
Cell Growth	DIVISION AND/OR ELONGATION	Zhang and Forde (1998) Science 279: 407-409. Coruzzi et al. U.S. Pat. No. 5,955,651

Other biological activities that can be modulated by the nitrogen responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Domain section above.

Nitrogen responsive genes are characteristically differentially transcribed in response to fluctuating nitrogen levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various nitrogen responsive genes in the aerial parts of a seedling at 2, 6, 9 and 12 hours after a plant was sprayed with a solution enriched with ammonium nitrate as compared to seedlings sprayed with water. The MA\_diff reports the changes in transcript levels of various nitrogen responsive genes in roots at 12 and 24 hours that were cut from seedlings transferred from a high to low potassium nitrate environment compared to control seedlings transferred to a high potassium nitrate environment.

The data from this time course reveal a number of types of nitrogen responsive genes and gene products, including "early responders," and "delayed nitrogen responders". Profiles of the individual categories of nitrogen responsive genes are shown in the Tables below together with examples of the kinds of associated biological activities that are modulated when the activities of one or more such genes vary in plants.

Low to High Ammonium Nitrate Experiment

Gene Expression Levels	Functional Category Of Gene	Physiological Consequences	Examples Of Gene Products
Upregulated Transcripts  (Level At 2h $\geq$ 6, 9 Or 12h) Or  (Level At 2h > 6, 9 Or 12h)	Early Responders To Nitrogen	- Perception Of Nitrogen  - Induced Nitrogen Uptake Into Cells  - Induction Of Nitrogen Response Transduction Pathways  - Initiation Of Specific Gene Transcription	- Transcription Factors  - Transporters  - Inhibitors Of Nitrogen Fixation  - Components Of Pathways Released From Repression  - Transaminases  - Amino Acid Biosynthetic Enzymes
Upregulated Transcripts  (Level At 2h < 6, 9, Or 12h)	Delayed Nitrogen Responders	- Maintenance Of High Nitrogen Metabolism And Growth	- Nitrogen Metabolic Pathway Enzymes  - Transaminases  - Amino Acid Biosynthetic Enzymes  - Factors Induced In Coordination And Control Of Central Carbon And Nitrogen Metabolism  - Cell Wall And Cell Growth- Promoting Pathway Enzymes  - Storage Proteins

Gene Expression Levels	Functional Category Of Gene	Physiological Consequences	Examples Of Gene Products
Down Regulated Transcripts (Level At 2h $\geq$ 6, 9 Or 12h) Or (Level At 6, 9 Or 12h > 2h)	- Early Responder Repressors Of Nitrogen Utilization Pathways  - Genes With Discontinued Expression Or Unstable Mrna Following Nitrogen Uptake	- Negative Regulation Of Nitrogen Utilization Pathways Released  - Pathways Of C And N Metabolism Required At Lower Levels Decline In Presence Of High Nitrogen	- Transcription Factors - Kinases And Phosphatases - Cytoskeletal Proteins Modulating Cell Structure - Chromatin Structure Regulatory Proteins - Metabolic Enzymes - Transporters - Proteins And Rna Turnover Systems

Gene Expression Levels	Functional Category Of Gene	Physiological Consequences	Examples Of Gene Products
Level At 2 Hours > 6,9 Or 12 Hours	<ul style="list-style-type: none"> <li>- Delayed Response</li> <li>Repressors Of Nitrogen Utilization Pathways</li> <li>Utilization Pathways</li> <li>- Genes With Discontinued Expression Or Unstable Mrna Following Nitrogen Uptake</li> </ul>	<ul style="list-style-type: none"> <li>- Negative Regulation Of Nitrogen Utilization Pathways Released</li> <li>- Pathways Of C And N Metabolism Required At Lower Levels</li> <li>Decline In Presence Of High Nitrogen</li> </ul>	<ul style="list-style-type: none"> <li>- Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>- Cytoskeletal Proteins Modulating Cell Structure</li> <li>- Chromatin Structure</li> <li>Regulatory Proteins</li> <li>- Metabolic Enzymes</li> <li>- Transporters</li> <li>- Protein And Rna Turnover Systems</li> </ul>

#### High to Low Potassium Nitrate Experiments

Gene Expression Levels	Functional Category Of Gene	Type Of Biological Activity	Examples Of Biochemical Activities Of Gene Products
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Gene Expression Levels	Functional Category Of Gene	Type Of Biological Activity	Examples Of Biochemical Activities Of Gene Products
Upregulated Transcripts (Level At 12h ~ 24h) (Level At 12h>24h)	Early Responders To Low Nitrate	<ul style="list-style-type: none"> <li>- Perception Of Low Nitrate</li> <li>- Nitrogen Uptake Into Cells</li> <li>- Low Nitrogen Signal Transduction Response Pathways</li> <li>- Initiation Of Specific Gene Transcription</li> <li>- Initiation Of Nitrogen Fixation</li> </ul>	<ul style="list-style-type: none"> <li>- Transcription Factors – Controlling Transcription</li> <li>- Transporters – Facilitating Transport</li> <li>- Cell Wall/Membrane Structure Determining Proteins</li> <li>- Kinases And Phosphatases- Regulating Signal Transduction Pathways</li> <li>- Cytoskeletal Proteins- Modulating Cell Structure</li> <li>- Chromatin Structure And/Or Dna Topology Proteins</li> <li>- Protein-Protein Interaction Participants</li> <li>- Metabolic Enzymes- Nitrogen Turnover Enzymes And Pathway Components</li> </ul>

Gene Expression Levels	Functional Category Of Gene	Type Of Biological Activity	Examples Of Biochemical Activities Of Gene Products
Upregulated Transcripts (Level 12h<24h)	Delayed Low Nitrate Responders	- Maintenance Of Low Nitrogen Response Pathways (See the Table Above)	- Transcription Factors – Controlling Transcription - Transporters – Facilitating Transport - Cell Wall/Membrane Structure Determining Proteins - Kinases And Phosphatases- Regulating Signal Transduction Pathways - Cytoskeletal Proteins- Modulating Cell Structure - Chromatin Structure And/Or Dna Topology Proteins - Protein-Protein Interaction Participants - Metabolic Enzymes- Nitrogen Turnover Enzymes And Pathway Components

Gene Expression Levels	Functional Category Of Gene	Type Of Biological Activity	Examples Of Biochemical Activities Of Gene Products
Down-Regulated Transcripts (Level At 12h~24h) (Level At 12h>24h)	- Early Repressor Responders To Low Nitrate  - Genes Whose Expression Is Discontinued Or Mrna Is UnsTable In Presence Of Low Nitrate	-Negative Regulation Of Low Nitrogen-Mediated Pathways And/Or Responses Released - Pathways In C And N Metabolism Required At Lower Levels Decline In The Presence Of Low Nitrate	- Transcription Factors Cell Wall/Membrane Structure Determining Proteins Factors For Promoting Protein Translation Kinases And Phosphatases - Cytoskeletal Proteins- Modulating Cell Structure - Protein And Rna Turnover Systems

Gene Expression Levels	Functional Category Of Gene	Type Of Biological Activity	Examples Of Biochemical Activities Of Gene Products
Down-Regulated Transcripts (Level At 12h<24h)	<ul style="list-style-type: none"> <li>- Delayed Repressor Responders To Low Nitrate</li> <li>- Genes Whose Expression Is Discontinued Or mRNA Is Unstable In Presence Of Low Nitrate</li> </ul>	<ul style="list-style-type: none"> <li>Negative Regulation Of Low Nitrogen-Mediated Pathways And/Or Responses Released Pathways In C And N</li> <li>Metabolism Required At Lower Levels Decline In The Presence Of Low Nitrate</li> </ul>	<ul style="list-style-type: none"> <li>- Transcription Factors Cell Wall/Membrane Structure Determining Proteins Factors For Promoting Protein Translation</li> <li>Kinases And Phosphatases</li> <li>- Cytoskeletal Proteins- Modulating Cell Structure</li> <li>- Protein And RNA Turnover Systems</li> <li>- Chromatin Structure And/Or DNA Topology Proteins</li> </ul>

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the nitrogen responsive genes when the desired sequence is operably linked to a promoter of a nitrogen responsive gene.

### III.D.2. CIRCADIAN RHYTHM (CLOCK) RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Often growth and yield are limited by the ability of a plant to tolerate stress conditions, including water loss. To combat such conditions, plant cells deploy a battery of responses that are controlled by an internal circadian clock, including the timed movement of cotyledons and leaves, timed movements in guard cells in stomata, and timed biochemical activities involved

with sugar and nitrogen metabolism. These responses depend on the functioning of an internal circadian clock, that becomes entrained to the ambient light/dark cycle, and a series of downstream signaling events leading to transcription independent and transcription dependent stress responses.

A functioning circadian clock can anticipate dark/light transitions and prepare the physiology and biochemistry of a plant accordingly. For example, expression of a chlorophyll a/b binding protein (CAB) is elevated before daybreak, so that photosynthesis can operate maximally as soon as there is light to drive it. Similar considerations apply to light/dark transitions and to many areas of plant physiology such as sugar metabolism, nitrogen metabolism, water uptake and water loss, flowering and flower opening, epinasty, germination, perception of season, and senescence.

Manipulation of one or more clock gene activities is useful to modulate the biological processes and/or phenotypes listed below. Clock responsive genes and gene products can act alone or in combination. Useful combinations include clock responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Circadian (relating to SMD 2344, SMD 2359, SMD 2361, SMD 2362, SMD 2363, SMD 2364, SMD 2365, SMD 2366, SMD 2367, SMD 2368, SMD 3242)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Circadian genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Circadian Genes Identified By Cluster Analyses Of Differential Expression

Circadian Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Circadian genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Circadian (relating to SMD 2344, SMD 2359, SMD 2361, SMD 2362, SMD 2363, SMD 2364, SMD 2365, SMD 2366, SMD 2367, SMD 2368, SMD 3242) of the MA\_diff table(s).

Circadian Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Circadian genes. A group in the MA\_clust is considered a Circadian pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Circadian Genes Identified By Amino Acid Sequence Similarity

Circadian genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Circadian genes. Groups of Circadian genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Circadian pathway or network is a group of proteins that also exhibits Circadian functions/utilities.

Such clock responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in daylength or in response to changes in light quality. Further, promoters of circadian (clock) responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by circadian or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the

circadian (clock) responsive genes when the desired sequence is operably linked to a promoter of a circadian (clock) responsive gene.

The expression of many genes is modulated by the clock. Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in response to the circadian rhythm clock at various times through the 24 hour cycle relative to the controls were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. The MA\_diff table reports the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent clock responsive genes.

### III.D.2.a. Use Of Circadian Rhythm (Clock) Responsive Genes To Modulate Phenotypes

Clock responsive genes and gene products are useful to or modulate one or more phenotypes including timing phenotypes, dormancy, germination, cotyledon opening, first leaves, juvenile to adult transition, bolting, flowering, pollination, fertilization, seed development, seed set, fruit drop, senescence, epinasty, biomass, fresh and dry weight during any time in plant life, such as maturation, number of flowers, seeds, branches, and/or leaves, seed yield, including number, size, weight, and/or harvest index, fruit yield, including number, size, weight, and/or harvest index, plant development, time to fruit maturity, cell wall strengthening and reinforcement, stress tolerance, drought tolerance, flooding tolerance, and uv tolerance.

To regulate any of the phenotype(s) above, activities of one or more of the clock responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant

utilizing the procedures described herein and the phenotypes can be screened for variants as in Anderson et al. (1997) Plant Cell 9: 1727-1743; Heintzen et al. (1997) Proc. Natl. Acad. Sci. USA 94: 8515-20; Schaffer et al. (1998) Cell 93:1219-1229; Somers et al. (1998) Development 125: 485-494; Somers et al. (1998) Science 282: 1488-1490; Wang and Tobin (1998) Cell 93: 1207-1217; Zhong et al. (1998) Plant Cell 10: 2005-2017; Sugano et al. (1998) Proc. Natl. Acad. Sci. USA 95: 11020-11025; Dowson-Day and Millar (1999) Plant J 17: 63-71; Green and Tobin (1999) Proc. Natl. Acad. Sci. USA 96: 4176-419; Staiger and Apel (1999) Mol. Gen. Genet. 261: 811-819; Strayer and Kay (1999) Curr. Opin. Plant Biol. 2:114-120; Strayer et. al. (2000) Science 289:768-771; Kreps et al. (2000) J Biol Rhythms (2000) 15:208-217; Nelson et al. (2000) Cell 101:331-340; Somers et al. (2000) Cell 101:319-329.

III.D.2.b. Use Of Active Clock Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the clock responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations above and included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Germination and seedling development	Cold, light and water modulated signal transduction pathways, receptors, kinases, PAS domain	Bognar et al. (1999) Proc. Natl. Acad. Sci. USA 96:14652-14657; Sugano et al (1999) Proc. Natl. Acad. Sci. USA 96:12362-12366; Dowson-Day and Millar (1999) Plant J 17: 63-71; Somers et al. (2000) Cell 101:319-329; Zhong et al. (1998) Plant Cell 10: 2005-2017
Growth transitions and flowering	Cold and light modulated signal transduction pathways, receptors, kinases, PAS domain	Nelson et al. (2000) Cell 101:331-340; Fowler et al. (1999) EMBO J. 18:4679-4688
Tuber formation	Cold and light modulated signal transduction pathways	Yanoovsky et al. (2000) Plant J. 23: 223-232
<u>METABOLISM</u>		
Lipid metabolism	Membrane lipid synthesis including omega-3 fatty acid desaturase, lipases, lipid transfer proteins	Bradley and Reddy (1997) J. Bacteriol. 179: 4407-4410; Martin, M et al. 1999 Europe J. Biochem 262: 283-290

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Sugar metabolism	Glycosylhydrolases, glycosyltransferases, amylases, sucrose synthase, CAB, Rubisco, light signal transduction	Liu et al. (1996) Plant Physiol. 112:43-51; Millar and Kay (1996) Proc Natl Acad Sci U S A 93:15491-15496; Wang et al. (1997) Plant Cell 9:491-507; Shinohara et al (1999) J. Biol. Chem. 273: 446-452
Nitrogen metabolism	Aminotransferases, arginase, proteases and vegetative storage proteins, aromatic amino acid synthesis	Bradley and Reddy (1997) J. Bacteriol. 179: 4407-4410
Photorespiration	Mitochondrial, chloroplast and peroxisomal photorespiratory enzymes, serine hydroxymethyl transferases, catalase	Zhong and McClung (1996) Mol. Gen. Genet. 251:196-203; McClung (1997) Free Radic. Biol. Med. 23:489-496; McClung et al. (2000) Plant Physiol. 123:381-392
Responses to Environmental Stress	Expression of genes involved in responses to drought, salt, UV	McClung (1997) Free Radic. Biol. Med 23:489-496; Shi et al. (2000) Proc. Natl. Acad. Sci. USA 97:6896-6901

Other biological activities that can be modulated by the clock responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Clock responsive genes are characteristically differentially transcribed in response to fluctuations in an entrained oscillator, which is internal to an organism and cell. The MA\_diff table(s) report(s) the changes in transcript levels of various clock responsive genes in a plant.

Profiles of clock responsive genes are shown in the table below with examples of which associated biological activities are modulated when the activities of one or more such genes vary in plants.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	Responders to circadian rhythm  Genes induced by rythm	<ul style="list-style-type: none"> <li>• Circadian rhythm perception</li> <li>• Metabolisms affected by Circadian rhythm</li> <li>• Synthesis of secondary metabolites and/or proteins</li> <li>• Modulation of clock response transduction pathways</li> <li>• Specific gene transcription initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Metabolic enzymes</li> <li>• Change in cell membrane structure and potential</li> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> <li>• Enzymes in lipid, sugar and nitrogen metabolism</li> <li>• Enzymes in photorespiration and photosynthesis</li> </ul>
Down-regulated transcripts	Responders to circadian rhythm.  Repressors of circadian "state" of metabolism	<ul style="list-style-type: none"> <li>• Negative regulation of circadian pathways released</li> <li>• Changes in</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or</li> </ul>

	<p>Genes repressed by rhythm</p> <p>Genes with discontinued expression or unsTable mRNA in presence of zinc</p>	<p>pathways and processes operating in cells</p> <ul style="list-style-type: none"><li>Changes in metabolism other than circadian pathways</li></ul>	<p>dephosphoryaltion (phosphatases)</p> <ul style="list-style-type: none"><li>Change in chromatin structure and/or DNA topology</li><li>Stability of factors for protein synthesis and degradation</li><li>Metabolic enzymes in light, sugar, lipid and nitrogen metabolism</li></ul>
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#### **USE OF PROMOTERS OF CLOCK RESPONSIVE GENES**

Promoters of Clock responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Clock responsive genes where the desired sequence is operably linked to a promoter of a Clock responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.D.3. BLUE LIGHT (PHOTOTROPISM) RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Phototropism is the orientation or growth of a cell, an organism or part of an organism in relation to a source of light. Plants can sense red (R), far-red (FR) and blue light in their environment and respond differently to particular ratios of these. For example, a low R:FR ratio

enhances cell elongation and favors flowering over leaf production, but blue light regulated cryptochromes also appear to be involved in determining hypocotyl growth and flowering time.

Phototropism of *Arabidopsis thaliana* seedlings in response to a blue light source is initiated by nonphototropic hypocotyl 1 (NPH1), a blue light-activated serine-threonine protein kinase, but the downstream signaling events are not entirely known. Blue light treatment leads to changes in gene expression. These genes have been identified by comparing the levels of mRNAs of individual genes in dark-grown seedlings, compared with in dark grown seedlings treated with 1 hour of blue light. Auxin also affects blue light phototropism. The effect of Auxin on gene expression stimulated by blue light has been explored by studying mRNA levels in a mutant of *Arabidopsis thaliana* nph4-2, grown in the dark and, treated with blue light for 1 hour compared with wild type seedlings treated similarly. This mutant is disrupted for Auxin-related growth and Auxin-induced gene transcription. Gene expression was studied using microarray technology.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) *Science* 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full-length cDNA and genomic sequence databanks, and the equivalent Ceres clones identified. The MA\_diff table(s) report(s) the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent blue light responsive genes and of those which are blue light responsive in the absence of nph4 gene activity. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Phototropism (relating to SMD 4188, SMD 6617, SMD 6619)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Blue Light genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Blue Light Genes Identified By Cluster Analyses Of Differential Expression

Blue Light Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Blue Light genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Phototropism (relating to SMD 4188, SMD 6617, SMD 6619) of the MA\_diff table(s).

Blue Light Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Blue Light genes. A group in the MA\_clust is considered a Blue Light pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Blue Light Genes Identified By Amino Acid Sequence Similarity

Blue Light genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Blue Light genes. Groups of Blue Light genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Blue Light pathway or network is a group of proteins that also exhibits Blue Light functions/utilities.

III.D.3.a. Use Of Blue Light Responsive Genes, Gene Components  
And Products To Modulate Phenotypes

Changes in blue light in a plant's surrounding environment result in modulation of many genes and gene products. Examples of such blue light response genes and gene products are shown in the REFERENCE and SEQUENCE Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While blue light responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different blue light responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a blue light responsive polynucleotides and/or gene product with other environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress and pathogen induced pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways.

III.D.3.b. Use Of Blue Light Responsive Genes, Gene Components  
And Products To Modulate Phenotypes

Blue light responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in blue light response concentration or in the absence of blue light responsive fluctuations. Further, promoters of blue light responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by blue light or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or

environmentally specific patterns as the blue light responsive genes when the desired sequence is operably linked to a promoter of a blue light responsive gene.

Blue light responsive genes and gene products can be used to alter or modulate one or more phenotypes including growth, roots (elongation or gravitropism) and stems (such as elongation), development of cell (such as growth or elongation), flower (including flowering time), seedling (including elongation), plant yield, and seed and fruit yield.

To regulate any of the phenotype(s) above, activities of one or more of the blue light responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Liscum and Briggs (1995, Plant Cell 7: 473-85), Vitha et al. (2000, Plant Physiol 122: 453-61), Stowe-Evance et al. (1998, Plant Physiol 118: 1265-75), Baum et al. (1999, PNAS USA 96: 13554-9), Huala et al. (1997) Science 278: 2120-2123), Kanegae et al. (2000, Plant Cell Physiol 41: 415-23), Khanna et al. (1999, Plant Mol Biol 39: 231-42), Sakai et al. (2000, Plant Cell 12: 225-36), Parks et al (1996, Plant Physiol 110: 155-62) and Janoudi et al. (1997, Plant Physiol 113: 975-79).

III.D.3.c. Use Of Blue Light Responsive Genes, Gene Components  
And Products To Modulate Biochemical Activities

The activities of one or more of the blue light responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cell Growth and Development	Cell Elongation <ul style="list-style-type: none"> <li>• Seedling</li> <li>• Stem</li> </ul>	Liscum and Briggs (1995) Plant Cell 7: 473-85
	• Root	Vitha et al. (2000) Plant Physiol 122: 453-61
Signalling	UV light Perception	Liscum and Briggs (1996) Plant Physiol 112: 291-96
	Far-red/Red light Perception	Parks et al. (1996) Plant Physiol 110: 155-62
	Phosphorylation of cellular and nuclear-localized proteins	Liscum and Briggs (1996) Plant Physiol 112: 291-96
	Activation and Synthesis of Transcription Factors	Sakae et al. (2000) Plant Cell 12: 225-36
	Ca+2 levels	Baum et al. (1999) PNAS USA 96: 13554-9 Pu and Robinson (1998) J Cell Sci 111: 3197-3207
	Auxin Concentration	Estelle (1998) Plant Cell 10: 1775-8 Reed et al. (1998) Plant Physiol 118: 1369-78
	Inter-photoreceptors	Janoudi et al. (1997) Plant Physiol 113: 975-79

Other biological activities that can be modulated by blue light response genes and their  
 Page 551 of 945

products are listed in the REF Tables. Assays for detecting such biological activities are described in the Domain section of the REF Table.

The specific genes modulated by blue light, in wild type seedlings and in the mutant deficient in transmitting Auxin effects are given in the Reference and Sequence Tables . The kinds of genes discovered and some of their associated effects are given in the Table below.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	Responders to no blue light in wild type or to blue light in mutant lacking Auxin effects	<ul style="list-style-type: none"> <li>• Blue light perception</li> <li>• Metabolism affected by blue light</li> <li>• Synthesis of secondary metabolites and/or proteins</li> <li>• Modulation of blue light transduction pathways</li> <li>• Specific gene transcription initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Transporters</li> <li>• Metabolic enzymes</li> <li>• Change in cell membrane structure and potential</li> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> </ul>
Down-regulated transcripts	Responders to no blue light in wild type or to blue light in mutants lacking Auxin effects	<ul style="list-style-type: none"> <li>• Blue light perception</li> <li>• Metabolism affected by blue light</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	Genes with discontinued expression or unsTable mRNA during response	<ul style="list-style-type: none"> <li>• Synthesis of secondary metabolites and/or proteins</li> <li>• Modulation of blue light transduction pathways</li> <li>• Specific gene transcription initiation</li> <li>• Changes in pathways and processes operating in cells</li> <li>• Changes in metabolic pathways other than phototropic blue light responsive pathways</li> </ul>	<p>dephosphorylation (phosphatases)</p> <ul style="list-style-type: none"> <li>• Change in chromatin structure and/or DNA topology</li> <li>• Stability factors for protein synthesis and degradation</li> <li>• Metabolic enzymes</li> </ul>

#### USE OF PROMOTERS OF BLUE LIGHT RESPONSIVE GENES

Promoters of Blue Light responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Blue Light responsive

genes where the desired sequence is operably linked to a promoter of a Blue Light responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.D.4 RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

There has been a recent and significant increase in the level of atmospheric carbon dioxide. This rise in level is projected to continue over the next 50 years. The effects of the increased level of carbon dioxide on vegetation are just now being examined, generally in large scale, whole plant (often trees) experiments. Some researchers have initiated physiological experiments in attempts to define the biochemical pathways that are either affected by and/or are activated to allow the plant to avert damage from the elevated carbon dioxide levels. A genomics approach to this issue, using a model plant system, allows identification of those pathways affected by and/or as having a role in averting damage due to the elevated carbon dioxide levels and affecting growth. Higher agronomic yields can be obtained for some crops grown in elevated CO<sub>2</sub>.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The U.S. Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing about 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in plants grown in higher CO<sub>2</sub> levels compared with plants grown at more normal CO<sub>2</sub> levels, were compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones were identified. The MA\_diff table reports the results of this analysis, indicating those

Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones cDNA sequences that change in response to CO<sub>2</sub>.

Examples of CO<sub>2</sub> responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff and MA\_clust tables. While CO<sub>2</sub> responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different CO<sub>2</sub> responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants.

Manipulation of one or more CO<sub>2</sub> responsive gene activities is useful to modulate the biological processes and/or phenotypes listed below. CO<sub>2</sub> responsive genes and gene products can act alone or in combination. Useful combinations include genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways.

CO<sub>2</sub> responsive genes and gene products can function to either increase or dampen the above phenotypes or activities. Further, promoters of CO<sub>2</sub> responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by CO<sub>2</sub> or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the CO<sub>2</sub> responsive genes when the desired sequence is operably linked to a promoter of a CO<sub>2</sub> responsive gene. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: CO2 (relating to SMD7561, SMD 7562, SMD 7261, SMD 7263, SMD 3710, SMD 4649, SMD 4650)). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

CO2 genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

CO2 Genes Identified By Cluster Analyses Of Differential Expression

CO2 Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of CO2 genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID CO2 (relating to SMD7561, SMD 7562, SMD 7261, SMD 7263, SMD 3710, SMD 4649, SMD 4650) of the MA\_diff table(s).

CO2 Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of CO2 genes. A group in the MA\_clust is considered a CO2 pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

CO2 Genes Identified By Amino Acid Sequence Similarity

CO2 genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis CO2 genes. Groups of CO2 genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a CO2 pathway or network is a group of proteins that also exhibits CO2 functions/utilities.

III.D.4.a. Use Of Co2 Responsive Genes To Modulate Phenotypes

CO<sub>2</sub> responsive genes and gene products are useful to or modulate one or more phenotypes including catabolism, energy generation, atp, etc., metabolism, carbohydrate synthesis, growth rate, whole plant, including height, flowering time, etc., organs, flowers, fruits, stems, leaves, roots, lateral roots, biomass, fresh and dry weight during any time in plant life, such as maturation; number, size, and weight of flowers; seeds; branches; leaves; total plant nitrogen content, amino acid/protein content of whole plant or parts, seed yield (such as number, size, weight, harvest index, and content and composition, e.g., amino acid, nitrogen, oil, protein, and carbohydrate); fruit yield; number, size, weight, harvest index; content and composition, e.g., amino acid, nitrogen, oil, protein, carbohydrate, water ; and photosynthesis (such as carbon dioxide fixation).

To improve any of the phenotype(s) above, activities of one or more of the CO<sub>2</sub> responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Saito et al. (1994, Plant Physiol. 106: 887-95), Takahashi et al (1997, Proc. Natl. Acad. Sci. USA 94: 11102-07) and Koprivova et al. (2000, Plant Physiol. 122: 737-46).

### **III.D.2. USE OF CO<sub>2</sub> RESPONSIVE GENES TO MODULATE BIOCHEMICAL ACTIVITIES**

The activities of one or more of the CO<sub>2</sub> responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cell Division	Cell Cycle Control Genes	Masle (2000) Plant Physiol. 122: 1399-1415
Starch Biosynthesis	Starch Biosynthesis Enzymes And Pathways	Ludewig et al., (1998) FEBS Lett. 429: 147-151
Photosynthesis	Photosynthetic Enzymes, e.g., Rubisco	Cheng et al., (1998) Plant Physiol 166: 715-723
Respiration	Energy Metabolism Pathways	Musgrave et al., (1986) Proc. Natl. Acad. Sci. USA 83: 8157-8161
CO <sub>2</sub> Uptake	Guard Cell Stomata Control Systems	Allen et al., Plant Cell (1999) 11(9): 1785-1798 Ichida et al., Plant Cell (1997) 9(10): 1843-1857 Hedrich et al., EMBO J (1993) 12(3): 897-901
Coordination Of Carbon And Nitrogen Metabolism	Light-Regulation Of Major Central Carbon And Nitrogen Metabolic Pathways To Coordinate Growth	Lam et al. (1998) Plant J. 16(3): 345-353 Lejay et al. (1999) Plant J. 18(5): 509-519; and Oliveira et al. (1999) Plant. Phys. 121: 301-309
	Carbohydrate And Nitrogen Control Of Carbohydrate And Organic Nitrogen Accumulation Pathways	Lam et al. (1998) supra; Lejay et al. (1999) supra; and Oliveira et al. (1999) supra

Other biological activities that can be modulated by the CO<sub>2</sub> responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

CO<sub>2</sub> responsive genes are characteristically differentially transcribed in response to fluctuating CO<sub>2</sub> levels or concentrations, whether internal or external to an organism or cell. The MA\_diff tables report the changes in transcript levels of various CO<sub>2</sub> responsive genes that are differentially expressed in response to high CO<sub>2</sub> levels.

Profiles of these different CO<sub>2</sub> responsive genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up Regulated Transcripts	Responders To Higher Levels Of CO <sub>2</sub>  Genes Induced By CO <sub>2</sub>	<ul style="list-style-type: none"><li>• Changes In Generation Of ATP</li><li>• Changes In Catabolism And Anabolism Enzymes and Pathways</li><li>• Activation Of Krebs Cycle</li><li>• Specific Gene Transcription Initiation</li><li>• Changes In Carbohydrate Synthesis</li><li>• Changes In Chloroplast Structure</li><li>• Changes In Photosynthesis</li><li>• Changes In Respiration</li></ul>	<ul style="list-style-type: none"><li>• Transporters</li><li>• Catabolic And Anabolic Enzymes</li><li>• Change In Cell Membrane Structure And Potential</li><li>• Kinases And Phosphatases</li><li>• Transcription Activators And Repressors</li><li>• Change In Chromatin Structure And/Or Localized DNA Topology</li></ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
			<ul style="list-style-type: none"> <li>• Redox Control</li> </ul>
Down-Regulated Transcripts	Responders To Higher Levels Of CO <sub>2</sub> Genes Repressed By CO <sub>2</sub>	<ul style="list-style-type: none"> <li>• Changes In Pathways And Processes Operating In Cells</li> <li>• Changes In Catabolism and Anabolism</li> <li>• Changes in Chloroplast Structure</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Change In Protein Structure By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li> <li>• Change In Chromatin Structure And/Or DNA Topology</li> <li>• Stability Of Factors For Protein Synthesis And Degradation</li> <li>• Metabolic Enzymes</li> </ul>

#### USE OF PROMOTERS OF CO<sub>2</sub> RESPONSIVE GENES

Promoters of CO<sub>2</sub> responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the CO<sub>2</sub> responsive genes where the desired sequence is operably linked to a promoter of a CO<sub>2</sub> responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such

promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.D.5. MITOCHONDRIA ELECTRON TRANSPORT (RESPIRATION) GENES, GENE COMPONENTS AND PRODUCTS**

One means to alter flux through metabolic pathways is to alter the levels of proteins in the pathways. Plant mitochondria contain many proteins involved in various metabolic processes, including the TCA cycle, respiration, and photorespiration and particularly the electron transport chain (mtETC). Most mtETC complexes consist of nuclearly-encoded mitochondrial proteins (NEMPs) and mitochondrially-encoded mitochondrial proteins (MEMPs). NEMPs are produced in coordination with MEMPs of the same complex and pathway and with other proteins in multi- organelle pathways. Enzymes involved in photorespiration, for example, are located in chloroplasts, mitochondria, and peroxisomes and many of the proteins are nuclearly-encoded. Manipulation of the coordination of protein levels within and between organelles can have critical and global consequences to the growth and yield of a plant. Genes which are manipulated by interfering with the mtETC have been characterized using microarray technology.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing about 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in the presence of the ETC inhibitor, 10 mM antimycin A compared with the control lacking antimycin A. were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. The MA\_diff table reports the results of this analysis,

indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones that represent respiration responsive genes.

Examples of genes and gene products that are responsive to antimycin A block of respiration are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff and MA\_clust tables. While respiration responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different respiration responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. Manipulation of one or more respiration responsive gene activities are useful to modulate the biological processes and/or phenotypes listed below.

Such respiration responsive genes and gene products can function to either increase or dampen the phenotypes or activities below. Further, promoters of respiration responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by respiration or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the respiration responsive genes when the desired sequence is operably linked to a promoter of a respiration responsive gene. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Mitochondria-Electron Transport (relating to SMD 8061, SMD 8063)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Mitochondria-Electron Transport genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Mitochondria-Electron Transport Genes Identified By Cluster Analyses Of Differential Expression

Mitochondria-Electron Transport Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Mitochondria-Electron Transport genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Mitochondria-Electron Transport (relating to SMD 8061, SMD 8063) of the MA\_diff table(s).

Mitochondria-Electron Transport Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Mitochondria-Electron Transport genes. A group in the MA\_clust is considered a Mitochondria-Electron Transport pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Mitochondria-Electron Transport Genes Identified By Amino Acid Sequence Similarity

Mitochondria-Electron Transport genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Mitochondria-Electron Transport genes. Groups of Mitochondria-Electron Transport genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Mitochondria-Electron Transport pathway or

network is a group of proteins that also exhibits Mitochondria-Electron Transport functions/utilities.

**III.D.5.a. Use Of Respiration Responsive Genes To Modulate Phenotypes**

Respiration responsive genes and gene products are useful to or modulate one or more phenotypes including catabolism; energy generation, ATP, etc.; growth rate; whole plant, including height, flowering time, etc.; organs; flowers; fruits; stems; leaves; roots, lateral roots; biomass; fresh and dry weight during any time in plant life, such as maturation; number, size, and weight of flowers; seeds; branches; leaves; total plant nitrogen content; amino acid/protein content of whole plant or parts; seed yield (such as number, size weight, harvest index, and content and composition, e.g., amino acid, nitrogen, oil, protein, and carbohydrate); fruit yield; number, size, weight, harvest index; content and composition, e.g., amino acid, nitrogen, oil, protein, carbohydrate, water; and photosynthesis (such as carbon dioxide fixation).

To improve any of the phenotype(s) above, activities of one or more of the respiration responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Saito et al. (1994, Plant Physiol. 106: 887-95), Takahashi et al (1997, Proc. Natl. Acad. Sci. USA 94: 11102-07) and Koprivova et al. (2000, Plant Physiol. 122: 737-46).

**III.D.5.b. Use Of Respiration-Responsive Genes To Modulate Biochemical Activities**

The activities of one or more of the respiration responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Respiration and energy-related processes	Mitochondrial Electron Transport Chain	Passam et al. (1973) Biochem Biophys. Acta 325: 54-61
	Alternative oxidase pathway	Saisho et al. (1997) Plant Mol. Biol. 35: 585-600 Vanlerberghe and McIntosh (1994) Plant Physiol. 105: 867-874
	ATP generation pathways ATP utilization pathways	Mahler and Cordes (1966) In Biological Chemistry, Harper and Row
	Chloroplast energy related pathways	Foyer et al. (1989) Arch. Biochem. Biophys. 268: 687-697 Mills et al. (1978) Biochem. Biophys. Acta 504: 298-309
	Peroxisome energy related pathways	Olsen (1998) Plant mol. Biol. 38: 163-89
	Cytoplasmic energy related pathways	Roberts et al. (1995) Febs Letters 373: 307-309
	Catabolism and Anabolism	Mahler and Cordes (1966) In Biological Chemistry, Harper and Row
	Aerobic versus anaerobic	Mahler and Cordes (1966) In

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	pathways	Biological Chemistry, Harper and Row
Coordination of Carbon and Nitrogen Metabolism	Light-regulation of major central carbon and nitrogen metabolic pathways to coordinate growth	Lam et al. (1998) Plant J. 16(3): 345-353 Lejay et al. (1999) Plant J. 18(5): 509-519; and Oliveira et al. (1999) Plant. Phys. 121: 301-309
	Carbohydrate and nitrogen control of carbohydrate and organic nitrogen accumulation pathways	Lam et al. (1998) Plant J. 16(3): 345-353 Lejay et al. (1999) Plant J. 18(5): 509-519; and Oliveira et al. (1999) Plant. Phys. 121: 301-309

Other biological activities that can be modulated by the respiration genes and gene products are listed in the REF Tables. Assays for detecting such biological activities are described in the Protein Domain table.

Respiration responsive genes are differentially expressed in response to inhibition of mitochondrial electron transport by antimycin A. The MA\_diff table reports the changes in transcript levels of various respiration responsive genes that are differentially expressed in response to this treatment.

Profiles of these different respiration genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	<p>Responders to inhibition of mitochondrial electron transport respiration</p> <p>Genes induced by inhibition of mitochondrial electron transport</p>	<ul style="list-style-type: none"> <li>Changes in generation of ATP</li> <li>Alternate oxidase induction</li> <li>Changes in catabolic and anabolic enzymes and pathways</li> <li>Specific gene transcription initiation</li> <li>Changes in electron transport proteins</li> </ul>	<ul style="list-style-type: none"> <li>Transporters</li> <li>Catabolic and anabolic enzymes</li> <li>Changes in cell and organelle membrane structures and potentials</li> <li>Kinases and phosphatases</li> <li>Transcription activators</li> <li>Change in chromatin structure and/or localized DNA topology</li> <li>Redox control</li> </ul>
Down-regulated transcripts	<p>Responders to inhibition of mitochondrial electron transport</p> <p>Genes repressed by inhibition of</p>	<ul style="list-style-type: none"> <li>Changes in ATP generating pathways</li> <li>Changes in pathways and processes operating</li> </ul>	<ul style="list-style-type: none"> <li>Transcription factors</li> <li>Change in protein structure by phosphorylation (kinases) or dephosphorylation</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	mitochondrial electron transport	<p>in cells</p> <ul style="list-style-type: none"><li>• Induction of aerobic pathways</li><li>• Changes in catabolism and anabolism</li></ul>	<p>(phosphatases)</p> <ul style="list-style-type: none"><li>• Transporters</li><li>• Catabolic and anabolic enzymes</li><li>• Changes in cell and organelle membrane structures and potentials</li><li>• Change in chromatin structure and/or localized DNA topology</li><li>• changes</li><li>• Stability factors for protein synthesis and degradation</li><li>• Metabolic enzymes</li></ul>
		<ul style="list-style-type: none"><li>• Changes in redox activities</li></ul>	<ul style="list-style-type: none"><li>• Changes in redox enzymes</li></ul>

## USE OF PROMOTERS OF RESPIRATION GENES

Promoters of Respiration genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Respiration genes where the desired sequence is operably linked to a promoter of a Respiration gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.D.6. PROTEIN DEGRADATION GENES, GENE COMPONENTS AND PRODUCTS**

One of the components of molecular mechanisms that operate to support plant development is the "removal" of a gene product from a particular developmental circuit once the substrate protein is not functionally relevant anymore in temporal and/or spatial contexts. The "removal" mechanisms can be accomplished either by protein inactivation (e.g., phosphorylation or protein-protein interaction) or protein degradation most notably via ubiquitination-proteasome pathway. The ubiquitination-proteasome pathway is responsible for the degradation of a plethora of proteins involved in cell cycle, cell division, transcription, and signal transduction, all of which are required for normal cellular functions. Ubiquitination occurs through the activity of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin-protein ligases (E3), which act sequentially to catalyze the attachment of ubiquitin (or other modifying molecules that are related to ubiquitin) to substrate proteins (Hochstrasser 2000, Science 289: 563). Ubiquitinated proteins are then routed to proteasomes for degradation processing [2000, Biochemistry and Molecular Biology of Plants, Buchanan, Gruissem, and Russel (eds), Amer. Soc. of Plant Physiologists, Rockville, MD]. The degradation mechanism can be selective and specific to the concerned target protein (Joazeiro and Hunter 2001, Science 289: 2061; Sakamoto et al., 2001, PNAS Online 141230798). This selectivity and specificity may be one of the ways that the activity of gene products is modulated.

III.D.6.a. Identification Of Protein Degradation Genes, Gene Components And Products

"Protein degradation" genes identified herein are defined as genes, gene components and products associated with or dependant on the ubiquitination – proteasome protein degradation process. . Examples of such "protein degradation" genes and gene products are shown in the Reference and Sequence Tables. The biochemical functions of the protein products of many of these genes are also given in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff and MA\_clust tables. Selected genes, gene components and gene products of the invention can be used to modulate many plant traits from architecture to yield to stress tolerance.

"Protein Degradation" Genes, Gene Components And Products Identified By Phenotypic Observations

"Protein degradation" genes herein were discovered and characterized from a much larger set of genes in experiments designed to find the genes associated with the increased number of lateral branches (and secondary inflorescences) that are formed per cauline node. In these experiments, "protein degradation" genes were identified using a mutant with these characteristics. The gene causing the changes was identified from the mutant gene carrying an inserted tag. The mutant plant was named 13B12-1 and the mutant was in the E2 conjugating enzyme gene of the ubiquitination process. Compared to "wild-type" parental plants, the mutant plants exhibited multiple lateral stems per node and multi-pistillated flowers. For more experimental detail, see Example section below.

Protein Degradation Genes, Gene Components And Products Identified By Differential Expression

"Protein degradation" genes were also identified by measuring the relative levels of mRNA products in the mutant plant 13B12-1 lacking the E2 conjugating enzyme versus a "wild-type" parental plant. Specifically, mRNAs were isolated from 13B12-1 and compared with mRNAs isolated from wild-type plants utilizing microarray procedures. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108451). For transcripts that had higher levels in

the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Protein Degradation genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Protein Degradation Genes Identified By Cluster Analyses Of Differential Expression

#### Protein Degradation Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Protein Degradation genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108451 of the MA\_diff table(s).

#### Protein Degradation Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Protein Degradation genes. A group in the MA\_clust is considered a Protein Degradation pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

#### Protein Degradation Genes Identified By Amino Acid Sequence Similarity

Protein Degradation genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and *Arabidopsis* Protein Degradation genes.

Groups of Protein Degradation genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Protein Degradation pathway or network is a group of proteins that also exhibits Protein Degradation functions/utilities.

These differentially expressed genes include genes associated with the degradation process and the genes whose expression is disturbed by the aberrant ubiquitination.

Examples of phenotypes, biochemical activities, and transcription profiles that can be modulated using these genes, gene components and gene products are described above and below.

III.D.6.b. Use Of "Protein Degradation" Genes, Gene Components  
And Products To Modulate Phenotypes

The "protein degradation" genes, their components and products of the instant invention are useful for modulating one or more processes required for post-translational modification (e.g., ubiquitination) and degradation or inactivation of substrate proteins and also the pathways and processes that are associated with protein inactivation that are important for either or all of the following: (i) cell proliferation; (ii) cell differentiation; and (iii) cell death. The "protein degradation" genes, gene components and gene products are useful to alter or modulate one or more phenotypes including cell proliferation and cell size.

The intracellular levels of many proteins are regulated by ubiquitin-proteasome proteolysis. Without proper regulation of protein levels, normal cell differentiation can be altered. Examples of cell differentiation and development can be modulated by the genes and gene products of this invention include root size (such as length of primary roots or length of lateral roots) and function; branching and stem formation (such as multiple pistils, multiple lateral stems or secondary inflorescence per caudine node, and internode length) and cell differentiation and/or development in response to hormones (such as Auxin).

Programmed cell death can result from specific and targeted degradation of critical substrate proteins (e.g., transcription factors, enzymes, and proteins involved in signal transduction). Thus, alteration of "protein degradation" genes, their gene products, and the corresponding substrate proteins that they are acting upon are useful to modulate the vigor and

yield of the plant overall as well as distinct cells, organs, or tissues. Traits that can be modulated by these genes and gene products include sterility or reproduction and seedling lethality.

**USES OF PLANTS THAT ARE MODIFIED AS DESCRIBED ABOVE**

Genes that control fundamental steps in regulatory pathways, such as protein inactivation, that in turn influence cascades and networks of other genes and processes are extremely useful. They and their component parts can be used selectively to manipulate development in specific cells, tissues and organs, including meristems when genes are designed to inactivate the normal genes only in specific cells, tissues and organs or to promote protein production where it is not normally produced. They can also be used to promote/control cell death.

Other "protein degradation" genes described here are components of the pathways determining organ identity and phenotypes. These and their component parts are also useful for modifying the characteristics of specific cells, tissues and organs when regulated appropriately. Thus "protein degradation" genes have wide utility for achieving the following: better plant survival by decreased lodging; better responses to high plant density; better stress tolerance; better animal (including human) nutrition values; improved dietary mineral nutrition; more vigor, growth rate and yield in terms of biomass; root/tuber yield (in terms of number, size, weight, or harvest index); content and composition, e.g. amino acid, jasmonate, oil, protein and starch; number of flowers; seed yield (e.g. number, size, weight, harvest index, content and composition, e.g. amino acid, jasmonate, oil, protein and starch); and fruit yield (e.g. number, size, weight, harvest index, post harvest quality, content and composition, e.g. amino acid, jasmonate, oil, protein and starch).

To regulate any of the phenotype(s) above, activities of one or more of the "protein degradation" genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. In addition, a synthetic molecule containing specific domains from "protein degradation" genes or gene product and/or in combination with other domains from gene products that are not necessarily related to protein degradation pathway can be constructed to target the degradation or inactivation of specific substrate proteins. As an example, a plant can be transformed according to Bechtold and

Pelletier (1998, *Methods. Mol. Biol.* 82:259-266) and/or screened for variants as in Winkler et al. (1998) *Plant Physiol* 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Dolan et al. (1993, *Development* 119: 71-84), Dolan et al. (1997, *Development* 124: 1789-98), Crawford and Glass (1998, *Trends Plant Science* 3: 389-95), Wang et al. (1998, *PNAS USA* 95: 15134-39), Gaxiola et al. (1998, *PNAS USA* 95: 4046-50), Apse et al. (1999, *Science* 285: 1256-58), Fisher and Long (1992, *Nature* 357: 655-60), Schneider et al. (1998, *Genes Devel* 12: 2013-21) and Hirsch (1999, *Curr Opin Plant Biol.* 2: 320-326).

**USE OF PROTEIN DEGRADATION GENES, GENE COMPONENTS AND PRODUCTS TO MODULATE BIOCHEMICAL ACTIVITIES**

One or more of the "protein degradation" genes and their components can be used to modulate biochemical or metabolic activities, processes and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth, Differentiation and Development	Auxin response	Schwechheimer et al, <i>Science</i> 292: 1379 (2001); Leyser et al, <i>Nature</i> 8: 161 (1993); Lasswell et al, <i>Plant Cell</i> 12: 2395 (2000)
	Photomorphogenesis via leaf cells and meristems	Schwechheimer et al, <i>Science</i> 292: 1379 (2001)
	Apical dominance via shoot meristems	Schwechheimer et al, <i>Science</i> 292: 1379 (2001)
	Lateral root development via root	Xie et al, <i>Genes Dev</i> 14: 3024

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	meristem	(2000)
	Hypocotyl, shoot elongation by hormone controlled process	Nagpal et al, Plant Physiol 123: 563 (2000)
Gene Expression and related cellular processes	mRNA stability	Johnson et al, PNAS 97: 13991 (2000);
	Gene activation	Pham and Sauer, 289: 2357 (2000)
	Cell division and cell cycle control in meristems	King et al, Cell 81: 279 (1995); Ciechanover et al, Cell 37: 57 (1984); Finley et al, Cell 37: 43 (1984); Robzyk et al, Science 287: 501 (2000)
	Chromatin remodeling	Roest et al, Cell 86: 799 (1996)
	Post-translational modification and organelle targeting of proteins	Biederer et al, Science 278: 1806 (1997)

Other biological activities that can be modulated by the "protein degradation" gene, gene components and products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

III.D.6.d. Use Of Protein Degradation Genes, Gene Components And Products To Modulate Transcription Levels Of Other Genes

The expression of many genes is "up regulated" or "down regulated" in the 13B12-1 mutant because some protein degradation genes and their products are integrated into complex networks that regulate transcription of many other genes. Some protein degradation genes are

therefore useful for modifying the transcription of other genes and hence complex phenotypes, as described above. Profiles of "protein degradation" genes are described in the Table below with associated biological activities. "Up-regulated" profiles are those where the gene produces mRNA levels that are higher in the 13B12-1 as compared to wild-type plant; and vice-versa for "down-regulated" profiles.

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
Up Regulated Transcripts	<ul style="list-style-type: none"> <li>• Genes induced as a consequence of mutant ubiquitination degradation system</li> <li>• Genes repressed by “protein degradation” system directly or indirectly</li> <li>• Genes repressed or mRNAs degraded as a consequence of mutant ubiquitination degradation process</li> </ul>	<ul style="list-style-type: none"> <li>• Shoot formation</li> <li>• Lateral stem, lateral and main inflorescence development</li> <li>• Internode elongation</li> <li>• Node determination and development</li> <li>• Root formation</li> <li>• Lateral root development</li> <li>• Proper response to Auxin and other growth regulators</li> <li>• Seed dormancy and seed development</li> <li>• Resistance to drought and other forms of stress</li> <li>• Secondary metabolite biosynthesis</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Activators and Repressors</li> <li>• Chromatin Structure and/or Localized DNA Topology determining proteins</li> <li>• Methylated DNA binding proteins</li> <li>• Kinases, Phosphatases</li> <li>• Signal transduction pathway proteins</li> <li>• Transporters</li> <li>• Metabolic Enzymes</li> <li>• Cell cycle checkpoint proteins</li> <li>• Cell Membrane Structure And Proteins</li> <li>• Cell Wall Proteins</li> <li>• Proteins involved in secondary</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
			<ul style="list-style-type: none"><li>metabolism</li><li>• Seed storage metabolism</li></ul>
Down Regulated Transcripts	<ul style="list-style-type: none"><li>• Genes activated by "protein degradation" systems directly or indirectly</li></ul>		

"Protein degradation" genes and gene products can be modulated alone or in combination as described in the introduction. Of particular interest are combination of these genes and gene products with those that modulate hormone responses and/or metabolism. Hormone responsive and metabolism genes and gene products are described in more detail in the sections above. Such modification can lead to major changes in plant architecture and yield.

USE OF PROMOTERS AND "PROTEIN DEGRADATION GENES, GENE COMPONENTS AND PRODUCTS"

Promoters of "protein degradation" genes, as described in the Reference tables, for example, can be used to modulate transcription of any polynucleotide, plant or non plant to achieve synthesis of a protein in association with production of the ubiquitination –proteasome pathway or the various cellular systems associated with it. Additionally such promoters can be used to synthesize antisense RNA copies of any gene to reduce the amount of protein product produced, or to synthesize RNA copies that reduce protein formation by RNA interference. Such modifications can make phenotypic changes and produce altered plants as described above.

**PATENT**  
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### III.D.7. CAROTENOGENESIS RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Carotenoids serve important biochemical functions in both plants and animals. In plants, carotenoids function as accessory light harvesting pigments for photosynthesis and to protect chloroplasts and photosystem II from heat and oxidative damage by dissipating energy and scavenging oxygen radicals produced by high light intensities and other oxidative stresses. Decreases in yield frequently occur as a result of light stress and oxidative stress in the normal growth ranges of crop species. In addition light stress limits the geographic range of many crop species. Modest increases in oxidative stress tolerance would greatly improve the performance and growth range of many crop species. The development of genotypes with increased tolerance to light and oxidative stress would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the soil environment.

In animals carotenoids such as beta-carotene are essential provitamins required for proper visual development and function. In addition, their antioxidative properties are also thought to provide valuable protection from diseases such as cancer. Modest increases in carotenoid levels in crop species could produce a dramatic effect on plant nutritional quality. The development of genotypes with increased carotenoid content would provide a more reliable and effective nutritional source of Vitamin A and other carotenoid derived antioxidants than through the use of costly nutritional supplements.

Genetic changes produced through DNA mutation in a plant can result in the modulation of many genes and gene products. Examples of such mutation altered genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor, nutritional content and seed yield.

While carotenoid synthesis and/or oxidative stress responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different carotenoid biosynthetic polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of an carotenoid synthesis or oxidative stress protective polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that

exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in a common pathway.

Such carotenoid synthesis/oxidative stress tolerance genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in light intensity or in the absence of osmotic fluctuations. They were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA products participate in carotenogenesis. These experiments made use of an *Arabidopsis* mutant (*Or*) having an accumulation of up to 500 times more beta-carotene than wild-type in non-photosynthetic tissues.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) *Science* 270: 467-70). The USA *Arabidopsis* Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in the mutant plant compared with wild type seedlings were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. MA\_diff Table reports the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent Carotenoid synthesis/oxidative stress tolerance responsive genes. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Cauliflower (relating to SMD 5329, SMD 5330)). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Carotenogenesis genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Carotenogenesis Genes Identified By Cluster Analyses Of Differential Expression

Carotenogenesis Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Carotenogenesis genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Cauliflower (relating to SMD 5329, SMD 5330) of the MA\_diff table(s).

Carotenogenesis Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Carotenogenesis genes. A group in the MA\_clust is considered a Carotenogenesis pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Carotenogenesis Genes Identified By Amino Acid Sequence Similarity

Carotenogenesis genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Carotenogenesis genes. Groups of Carotenogenesis genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a

Carotenogenesis pathway or network is a group of proteins that also exhibits Carotenogenesis functions/utilities.

III.D.7.a. Use Of Carotenoid Synthesis/Oxidative Stress Tolerance Responsive Genes, Gene Components And Products To Modulate Phenotypes

Carotenoid synthesis/oxidative stress tolerance genes and gene products are useful to or modulate one or more phenotypes including growth rate; whole plant, including height, flowering time, etc.); seedling; organ (such as stem, leaves, roots, flowers, fruits, or seed yield, size, or weight); seed development; embryo; germination; cell differentiation; chloroplasts; plant nutrition; uptake and assimilation of organic compounds; uptake and assimilation of inorganic compounds; animal (including human) nutrition; improved dietary mineral nutrition; stress responses; drought; cold; and osmotic.

To improve any of the phenotype(s) above, activities of one or more of the Carotenoid synthesis/oxidative stress tolerance genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Friedrich, (1999, JAMA 282: 1508), Kumar et al. (1999, Phytochemistry 51: 847-51), La Rocca et al. (2000, Physiologia Plantarum 109: 51-7) and Bartley (1994, In: Ann Rev Plant Physiol Plant Molec Biol, Jones and Somerville, eds, Annual Reviews Inc, Palo Alto, CA).

III.D.7.b. Use Of Carotenoid Synthesis/Oxidative Stress Tolerance Responsive Genes, Gene Components And Products To Modulate Biochemical Activities

The activities of one or more of the carotenoid synthesis/oxidative stress tolerance genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth , Differentiation and Development	Chloroplast biosynthesis	Kumar et al. (1999) Phytochemistry 51: 847-51 Fraser et al. (1994) Plant Physiol 105: 405-13
Metabolism	Carotenoid biosynthesis	Kumar et al. (1999) Phytochemistry 51: 847-51
	Herbicide resistance	La Rocca et al. (2000) Physiogia Plantarum 109: 51-57
	Regulate abscisic acid levels	Tan et al. (1997) PNAS USA 94: 12235-40
	Drought, cold and osmotic tolerance	Tan et al. (1997) PNAS USA 94: 12235-40

Other biological activities that can be modulated by the Carotenoid synthesis, oxidative stress tolerance genes and gene products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Protein Domain table.

Profiles of these different carotenoid synthesis/oxidative stress tolerance responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	Genes induced during carotenoid synthesis/ oxidative stress tolerance activity	<ul style="list-style-type: none"> <li>• Gene Repression/Induction activity</li> <li>• Cell cycle progression</li> <li>• Chromatin condensation</li> <li>• Synthesis of metabolites and/or proteins</li> <li>• Modulation of transduction pathways</li> <li>• Specific gene transcription initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Transporters</li> <li>• Metabolic enzymes</li> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> </ul>
Down-regulated transcripts	Genes repressed during carotenoid synthesis/oxidative stress tolerance activity  Genes with discontinued expression or unsTable mRNA in conditions of reduced carotenoid	<ul style="list-style-type: none"> <li>• Gene repression/induction activity</li> <li>• Changes in pathways and processes operating in cells</li> <li>• Changes in metabolism other than carotenoid synthesis/oxidative</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or dephosphorylation (phosphatases)</li> <li>• Change in chromatin structure and/or</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	synthesis/oxidative stress tolerance	stress tolerance	<ul style="list-style-type: none"><li>• DNA topology</li><li>• Stability of factors for protein synthesis and degradation</li><li>• Metabolic enzymes</li></ul>

#### USE OF PROMOTERS OF CAROTENOGENESIS RESPONSIVE GENES

Promoters of Carotenogenesis responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Carotenogenesis responsive genes where the desired sequence is operably linked to a promoter of a Carotenogenesis responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.D.8. VIABILITY GENES, GENE COMPONENTS AND PRODUCTS**

Plants contain many proteins and pathways that when blocked or induced lead to cell, organ or whole plant death. Gene variants that influence these pathways can have profound effects on plant survival, vigor and performance. The critical pathways include those concerned with metabolism and development or protection against stresses, diseases and pests. They also include those involved in apoptosis and necrosis. The applicants have elucidated many such genes and pathways by discovering genes that when inactivated lead to cell or plant death.

Herbicides are, by definition, chemicals that cause death of tissues, organs and whole plants. The genes and pathways that are activated or inactivated by herbicides include those that

cause cell death as well as those that function to provide protection. The applicants have elucidated these genes.

The genes defined in this section have many uses including manipulating which cells, tissues and organs are selectively killed, which are protected, making plants resistant to herbicides, discovering new herbicides and making plants resistant to various stresses.

### III.D.8.a. Identification Of Viability Genes, Gene Components And Products

Viability genes identified here are defined as genes, gene components and products capable of inhibiting cell, tissue, organ or whole plant death or protecting cells, organs and plants against death and toxic chemicals or stresses. Examples of such viability genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff, MA\_clust, Knock-in and Knock-out tables. The biochemical functions of the protein products of many of these genes determined from comparisons with known proteins are also given in the Reference tables.

#### Viability Genes, Gene Components And Products Identified By Phenotypic Observations

These genes were discovered and characterized from a much larger set of genes by experiments designed to find genes that cause serious disturbances in progeny survival, seed germination, development, embryo and/or seedling growth. In these experiments, viability genes were identified by either (1) ectopic expression of a cDNA in a plant or (2) mutagenesis of a plant genome. The plants were then cultivated and one or more of the following phenotypes, which varied from the parental wild-type was observed:

- A. Gametophytic loss of progeny seedlings (detected from a parent on the basis of a linked herbicide resistance gene showing abnormal segregation ratios, as revealed by treating with herbicide)
- B. Embryo death, resulting in some cases to loss of seed
- C. Pigment variation in cotyledons and leaves, including absence of chlorophyll, which leads to seedling death.

#### 1. Abinos

2. Yellow/greens

- D. Cotyledons produced but no or few leaves and followed by seedling death.
- E. Very small plantlets

The genes identified in these experiments are shown in Tables X.

Viability Genes, Gene Components And Products Identified By Differential Expression

Viability genes were also identified from a much larger set of genes by experiments designed to find genes whose mRNA products changed in concentration in response to applications of different herbicides to plants. Viability genes are characteristically differentially transcribed in response to fluctuating herbicide levels or concentrations, whether internal or external to an organism or cell. The MA\_diff Table reports the changes in transcript levels of various viability genes in entire seedlings at 0, 4, 8, 12, 24, and 48 hours after a plant was sprayed with a Hoagland's nutrient solution enriched with either 2,4 D (Trimec), Glean, Grassgetter, Roundup, or Finale herbicides as compared to seedlings sprayed with Hoagland's solution only.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108467, 107871, 107876, 108468, 107881, 108465, 107896, 108466, 107886, 107891, 108501). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Viability genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a "+" or "-" indication.

Viability Genes Identified By Cluster Analyses Of Differential Expression

Viability Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in

the same pathway or network.

A pathway or network of Viability genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108467, 107871, 107876, 108468, 107881, 108465, 107896, 108466, 107886, 107891, 108501 of the MA\_diff table(s).

Viability Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Viability genes. A group in the MA\_clust is considered a Viability pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Viability Genes Identified By Amino Acid Sequence Similarity

Viability genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Viability genes. Groups of Viability genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Viability pathway or network is a group of proteins that also exhibits Viability functions/utilities.

It is assumed that those gene activity changes in response to the toxic herbicides are either responsible, directly or indirectly, for cell death or reflect activation of defense pathways. These genes are therefore useful for controlling plant viability.

Examples of phenotypes, biochemical activities, or transcript profiles that can be modulated using selected viability gene components are described above and below.

III.D.8.b. Use Of Viability Genes, Gene Components And Products To Modulate Phenotypes

Deficiencies in viability genes can cause cell death at various rates and under various conditions. Viability genes can be divided into two classes; (1) those that lead to cell death under permissive growth conditions and (2) those that cause cell demise under restrictive conditions. Examples of the first class are viability genes which encode toxins or which

participate in the programmed cell death pathway(s). Disruption of metabolic pathways, such as amino acid synthesis, may not cause death when the cell is supplemented with appropriate amino acids, but can cause death under more restrictive conditions.

Some deficiencies in viability genes identified cause the organism as a whole to die, while other genes cause death only of a specific subset of cells or organs. For example, genes identified from embryo viability phenotypes can cause an entire organism to die. In contrast, genes characterized from gametophytic lethals may inhibit cell growth only in a select set of cells. In addition, some viability genes may not cause an immediate demise. A seedling lethal phenotype is one such example, where a seed germinates and produces cotyledons but the plant dies before producing any true leaves. Yellow-green pigment mutants provide yet another set of examples. In some cases, the plant produces a number of yellow-green leaves but dies before producing any seed, due in part, to the necessity to produce chlorophyll in functioning chloroplasts to fix CO<sub>2</sub>.

Viability genes, in which mutational deficiencies lead to death, carry no duplicates in the haploid plant genome. They thus may be especially likely to promote viability and vigor when expressed more optimally in a plant, in specific tissues or throughout the plant.

Proteins which lead to death when inactivated, and other proteins in the pathways in which they act, are potential targets for herbicides. In this kind of application, chemicals specifically capable of interacting with such proteins are discovered. Typically, this could be done by designing a gene involving the relevant viability gene, that also facilitates a rapid easily measured assay for the functioning of the protein product, and treating plants containing the new genes with the potential herbicides. Those chemicals specifically interfering with the protein activity can then easily be selected for further development.

Genes whose products interact directly with a herbicide can also be modified such that the herbicide no longer inactivates the protein. Such genes are useful for making herbicide resistant plants, valuable in agriculture.

Many of the genes activated or inactivated by the herbicides define genes involved in the pathways that protect the plant against damage and stresses. These genes and gene components, especially those regulating such pathways, are especially useful for enhancing the ability of

plants to withstand specific stresses, including herbicides. [See the sections on Stress responsive genes, gene components and products.]

Genes that cause cellular death can be used to design new genes that cause death of specific cells and tissues and hence new valuable products. For example, activation of genes causing death in cells specifying seeds can be used to produce fruits lacking seeds. They can also be used to prevent cell death by pathogens and pests.

The genes and gene components of the instant invention are useful to modulate one or more processes that affect viability and vigor at the (1) cellular level; (2) organelle level; (3) organ level; or (4) overall organism level.

Phenotypes that are modulated by these genes and gene components include (1) at the cellular level: cell size, cell differentiation, cell division, cell longevity, cell position, and cytotoxins; (2) at the organelle level: chloroplasts and/or mitochondria; (3) at the organ level: flower number or size; seed size, number or composition (amino Acid, carbohydrates, lipid, and secondary metabolites); fruit size, number, or composition (amino Acid, carbohydrates, lipid, and secondary metabolites); fruit drop, fruit ripening; leaf (size, composition, amino acid, carbohydrates, lipid, and secondary metabolites, photoefficiency, abscission, or senescence); stem; or root; and (4) at the overall organism level: vigor (e.g. increased biomass), stress tolerance (e.g. cold, drought, heat, herbicide, oxidative, and salt); and pathogen resistance

To regulate any of the phenotype(s) above, activities of one or more of the viability genes or gene products can be modulated in an organism and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (*Methods. Mol. Biol.* 82:259-266 (1998)) and/or screened for variants as in Winkler et al., *Plant Physiol.* 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed.

III.D.8.c. Use Of Viability Genes, Gene Components And Products To Modulate Biochemical Activities

The viability genes, their components and/or products can be used to modulate processes, biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Amino Acid Synthesis	Aceto -lactate synthase	Hershey et al. (1999) Plant Mol. Biol. 40, 795-806
Cell Wall Synthesis	Cellulose synthase	Peng et al. (2001) Plant Physiol. 126, 981-982 Kawagoe and Delmer (1997) Genet Eng. 19, 63-87
Nucleotide Synthesis	Coenzyme A biosynthesis	Kupke et al. (2001) J. Biol. Chem. 276, 19190-19196
Lipid Synthesis	Oleosin biosynthesis	Singh et al. (2000) Biochem. Soc. Trans. 28, 925-927 Zou et al. (1996). Plant Mol. Biol. 31, 429-433
Hormone Signaling Pathways	Brassinolide and light signal transduction	Kang et al. (2001) Cell 105, 625-636
Hormone Biosynthesis	Cytokinin biosynthesis	Takei et al, (2001) J. Biol. Chem. 276, 26405-26410
Secondary Metabolites	• Carotenoid biosynthesis	Estevez et al. (2001) J. Biol. Chem. 276, 22901-22909 Carol and Kuntz (2001) Trendy Plant Sci. 6, 31-36 Pogson and Rissler (2001) Phil. Trans. Roy. Soc. Lord.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
		B 355, 1395-1400
Clearing of Toxic Substances	Ubiquitination	
Growth, Differentiation And Development	<ul style="list-style-type: none"> <li>• Farnesylation</li> <li>• Nitrogen Metabolism</li> </ul>	Pei et al (1998) Science 282: 287-290; Cutler et al. (1996) Science 273: 1239 Goupil et al (1998) J Exptl Botany 49:1855-62
Water Conservation And Resistance To Drought And Other Related Stresses	<ul style="list-style-type: none"> <li>• Stomatal Development And Physiology</li> <li>• Stress Response Pathways</li> <li>• Inhibition Of Ethylene Production Under Low Water Potential</li> <li>• Proline And Other Osmolite Synthesis And Degradation</li> </ul>	Allen et al. (1999) Plant Cell 11: 1785-1798 Li et al. 2000 Science 287: 300-303 Burnett Et Al 2000. J. Exptl Botany 51: 197-205 Raschke (1987) In: Stomatal Function Zeiger et al. Eds., 253-279 Bush And Pages (1998) Plant Mol. Biol. 37: 425-35 Spollen Et Al (2000) Plant Physiol. 122:967-976 Hare et al. (1998) Plant, Cell And Environment 21:535-553; Hare et al. (1999) J. Exptl. Botany 50:413-434

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Programmed cell death	<ul style="list-style-type: none"> <li>• Proteases</li> <li>• DNA endonucleases</li> <li>• Mitochondriae uncoupling proteins</li> </ul>	Kamens et al. (1995) <i>J. Biol. Chem.</i> 270, 15250-15256 Wang et al. (2001) <i>Anticancer Res.</i> 21, 1789-1794 Drake et al. (1996) <i>Plant Mol. Biol.</i> 304, 755-767 Mittler and Lam (1995) <i>Plant Cell</i> 7, 1951-1962 Mittler and Lam (1995) <i>Plant Physiol.</i> 108, 489-493 Thelen and Northcote (1989) <i>Planta</i> 179, 181-195 Hanak and Jezek (2001) <i>FEBS Lett.</i> 495, 137-141
	<ul style="list-style-type: none"> <li>• Plasmalemma and Tonoplast Ion Channel Changes</li> <li>• Ca<sup>2+</sup> Accumulation</li> <li>• K<sup>+</sup> Efflux</li> </ul>	Macrobbie (1998) <i>Philos Trans R Soc Lond B Biol Sci</i> 353: 1475-88; Li et al (2000) <i>Science</i> 287:300-303; Barkla et al. (1999) <i>Plant Physiol.</i> 120:811-819 Lacombe et al. (2000) <i>Plant Cell</i> 12: 837-51; Wang et al. (1998) <i>Plant Physiol.</i> 118:1421-1429; Shi et al. (1999) <i>Plant Cell</i> 11: 2393-

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	<ul style="list-style-type: none"><li>Activation Of Kinases And Phosphatases</li></ul>	2406 Gaymard et al. (1998) Cell 94:647-655 Jonak et al. (1996) Proc. Natl. Acad. Sci 93: 11274-79; Sheen (1998) Proc. Natl. Acad. Sci. 95: 975-80; Allen et al. (1999) Plant Cell 11: 1785-98

Other biological activities that can be modulated by the viability genes, their components and products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

**III.D.8.d. Use Of Viability Genes, Gene Components And Products To Modulate Transcript Levels Of Other Genes**

The expression of many genes is “up regulated” or “down regulated” following herbicide treatment and also in the leaf mutants, because some “viability” genes and their products are integrated into complex networks that regulate transcription of many other genes. Some “viability genes” are therefore useful for modifying the transcription of other genes and hence complex phenotypes, as described above. The data from differential expression experiments can be used to identify a number of types of transcript profiles of “viability genes”, including “early responders,” and “delayed responders”, “early responder repressors” and “delayed repressors”. Profiles of these different types responsive genes are shown in the Table below together with examples of the kinds of associated biological activities. “Up-regulated” profiles are those where the mRNA transcript levels are higher in the herbicide treated plants as compared to the

untreated plants. "Down-regulated" profiles represent higher transcript levels in the untreated plant as compared to the herbicide treated plants.

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
Up Regulated Transcripts  (Level At 4 Hr $\geq$ 0 Hr) or  (Level At 4 Hr > 0 Hr)	<ul style="list-style-type: none"> <li>• Early Responders To:                     <ul style="list-style-type: none"> <li>• Gluphosinate</li> <li>• Chlorsulfuron</li> <li>• Glyphosate and/or 2, 4-D</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Suppression of cell, tissue, organ or plant death following:</li> <li>• Herbicide treatment or under stress</li> <li>• Activation of cell, tissue, organ or plant death following:</li> <li>• Herbicide treatment or under stress</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Transporters</li> <li>• Change In Cell Membrane Structure</li> <li>• Kinases And Phosphatases</li> <li>• Germins, Germin-like proteins, Calcium-binding proteins and H<sub>2</sub>O<sub>2</sub> generating and H<sub>2</sub>O<sub>2</sub> neutralizing proteins.</li> <li>• Transcription Activators</li> <li>• Change In Chromatin Structure And/Or Localized DNA Topology</li> <li>• Annexins, cell wall</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
			structural proteins
Up Regulated Transcripts (Level At 4 Hr < 12 Hr)	Delayed Responders to Gluphosinate, Chlorsulfuron, Glyphosate and/or 2, 4-D	<ul style="list-style-type: none"> <li>• Suppression of cell, tissue, organ or plant death following:</li> <li>• Herbicide treatment or under stress</li> <li>• Activation of cell, tissue, organ or plant death following:</li> <li>• Herbicide treatment or under stress</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Specific Factors (Initiation And Elongation) For Protein Synthesis</li> <li>• Lipid transfer proteins</li> <li>• Myrosinase-binding proteins</li> <li>• Sugar interconverting enzymes</li> <li>• Maintenance Of mRNA Stability</li> <li>• Maintenance Of Protein Stability</li> <li>• Maintenance Of Protein-Protein Interaction</li> <li>• Protein translocation factors</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
			<ul style="list-style-type: none"> <li>• RNA-binding proteins</li> <li>• Centromere and cytoskeleton proteins</li> <li>• Lipases</li> <li>• Zn/Cu transporters</li> <li>• Cell wall structural proteins</li> </ul>
Down-Regulated Transcripts (Level At 0 Hr $\leq$ 4 Hr) or (Level At 0 Hr > 4 Hr)	Early Responder Repressors Of Stress Response State Of Metabolism  Genes With Discontinued Expression Or Unstable mRNA In Presence Of Herbicide or Abiotic Stress	<ul style="list-style-type: none"> <li>• Suppression of cell, tissue, organ or plant death following:                             <ul style="list-style-type: none"> <li>• Herbicide treatment or under stress</li> <li>• Activation of cell, tissue, organ or plant death following:                                     <ul style="list-style-type: none"> <li>• Herbicide treatment or</li> </ul> </li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Change In Protein Structure By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li> <li>• Change In Chromatin Structure And/Or DNA Topology</li> <li>• H<sub>2</sub>O<sub>2</sub> neutralizing</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
		<ul style="list-style-type: none"> <li>under stress</li> <li>• Zn/Cu transporters</li> <li>• Cell wall structural proteins</li> </ul>	<ul style="list-style-type: none"> <li>proteins</li> <li>• Neutralizing proteins including SOD and GST</li> </ul>
Down-Regulated Transcripts (Level At 4 Hr > 12 Hr)	<p>Delayed Responder Repressors Of ABA Function State Of Metabolism</p> <p>Genes With Discontinued Expression Or Unstable</p>	<ul style="list-style-type: none"> <li>• Suppression of cell, tissue, organ or plant death following:                     <ul style="list-style-type: none"> <li>• Herbicide treatment or under stress</li> <li>• Activation of cell, tissue, organ or plant death following:</li> <li>• Herbicide treatment or under stress</li> </ul> </li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Kinases And Phosphatases</li> <li>• Stability Of Factors For Protein Synthesis And Degradation</li> <li>• Amino Acid biosynthesis proteins including aspargive synthase</li> <li>• Ca-binding proteins</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES WHOSE TRANSCRIPTS ARE CHANGED	PHYSIOLOGICAL CONSEQUENCES OF MODIFYING GENE PRODUCT LEVELS	EXAMPLES OF BIOCHEMICAL ACTIVITIES WHOSE TRANSCRIPTS ARE CHANGED
	mRNA In Presence Of herbicide or Abiotic Stress		<ul style="list-style-type: none"><li>• Lipid biosynthesis proteins</li><li>• Lipases</li><li>• Zn/Cu transporters</li><li>• Cell wall structural proteins</li></ul>

While viability modulating polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development.

#### USE OF PROMOTERS OF VIABILITY GENES, GENE COMPONENTS AND PRODUCTS

Promoters of viability genes can include those that are induced by (1) destructive chemicals, e.g. herbicides, (2) stress, or (3) death. These promoters can be linked operably to achieve expression of any polynucleotide from any organism. Specific promoters from viability genes can be selected to ensure transcription in the desired tissue or organ. Proteins expressed under the control of such promoters can include those that can induce or accelerate death or those that can protect plant cells from organ death. For example, stress tolerance can be increased by using promoters of viability genes to drive transcription of cold tolerance proteins, for example. Alternatively, promoters induced by apoptosis can be utilized to drive transcription of antisense constructs that inhibit cell death.

#### **III.D.9. HISTONE DEACETYLASE (AXEL) RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

The deacetylation of histones is known to play an important role in regulating gene expression at the chromatin level in eukaryotic cells. Histone deacetylation is catalyzed by proteins known as histone deacetylases (HDacs). HDacs are found in multisubunit complexes that are recruited to specific sites on nuclear DNA thereby affecting chromatin architecture and target gene transcription. Mutations in plant HDAc genes cause alterations in vegetative and reproductive growth that result from changes in the expression and activities of HDAc target genes or genes whose expression is governed by HDAc target genes. For example, transcription factor proteins control whole pathways or segments of pathways and proteins also control the activity of signal transduction pathways. Therefore, manipulation of these types of protein levels is especially useful for altering phenotypes and biochemical activities.

Manipulation of one or more HDAc gene activities is useful to modulate the biological activities and/or phenotypes listed below. HDAc genes and gene products can act alone or in combination. Useful combinations include HDAc genes and/or gene products with similar biological activities, or members of the same, co-regulated or functionally related biochemical pathways. Such HDAc genes and gene products can function to either increase or dampen these phenotypes or activities.

Examples of genes whose expression is affected by alterations in HDAc activity are shown in the Reference and Sequence Tables. These genes and/or gene products are responsible for effects on traits such as inflorescence branching and seed production. They were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA products are affected by a decrease in HDAc gene activity. These experiments made use of an *Arabidopsis* mutant having severely reduced mRNA levels for the histone deactylase gene AtHDAC1.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The *Arabidopsis* Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing 10,000 non-redundant ESTs, selected from 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full-length cDNA and genomic sequence databanks, and identical Ceres clones identified. MA\_diff table reports the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which are HDAC genes. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Axel (relating to SMD 6654, SMD 6655)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Histone Deacetylase genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Histone Deacetylase Genes Identified By Cluster Analyses Of Differential Expression

Histone Deacetylase Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Histone Deacetylase genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Axel (relating to SMD 6654, SMD 6655) of the MA\_diff table(s).

Histone Deacetylase Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Histone Deacetylase genes. A group in the MA\_clust is considered a Histone Deacetylase pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Histone Deacetylase Genes Identified By Amino Acid Sequence Similarity

Histone Deacetylase genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Histone Deacetylase genes. Groups of Histone Deacetylase genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Histone Deacetylase pathway or network is a group of proteins that also exhibits Histone Deacetylase functions/utilities.

III.D.9.a. Use Of Hdac Genes, Gene Components And Products To Modulate Phenotypes

HDAC genes and gene products are useful to or modulate one or more phenotypes including growth rate; whole plant, including height, flowering time, etc.; seedling; organ; seed development; embryo; germination, and cell differentiation.

To improve any of the phenotype(s) above, activities of one or more of the HDAC genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Wu et al. (2000,, Plant J 22: 19-27), Hu et al. (2000, J Biol Chem 275: 15254-64), Johnson and Turner (1999, Semin Cell Dev Biol 10: 179-88), Koyama et al. (2000, Blood 96: 1490-5), Wu et al. (2000, Plant J 22: 19-27), Li (1999, Nature Genetics 23: 5-6), Adams et al. (2000, Development 127: 2493-2502) and Lechner et al. (2000, Biochemistry 39: 1683-92).

III.D.9.b. Use Of Hdac Development Genes, Gene Components And Products To Modulate Biochemical Activities

The activities of one or more of the HDAC genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth, Differentiation And Development	<ul style="list-style-type: none"><li>• Cell Differentiation</li><li>• Cell Cycle Progression</li></ul>	Koyama et al. (2000) Blood 96: 1490-5 Hu et al. (2000) J Biol Chem 275: 15254-64
Metabolism	<ul style="list-style-type: none"><li>• Chromatin Structure</li></ul>	Hu et al. (2000) J Biol Chem

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	<ul style="list-style-type: none"> <li>• Gene Transcription And Chromatin Assembly</li> </ul>	275: 15254-64 Johnson and Turner (1999) <i>Semin Cell Dev Biol</i> 10: 179-88
Reproduction And Seed Development	<ul style="list-style-type: none"> <li>• Seed Development</li> <li>• Seed Germination</li> <li>• Independent Embryo Fertilization</li> <li>• Fertilization Independent Seed Development</li> <li>• Megagametogenesis</li> </ul>	Wu et al. (2000) <i>Plant J</i> 22:19-27 Lechner et al. (2000) <i>Biochemistry</i> 39: 1683-92 Ohad et al. (1996) <i>PNAS USA</i> 93: 5319-24 Chaudhury et al. (1997) <i>PNAS USA</i> 94: 4222-28 Christensen et al. (1997) <i>Sex Plant Reproduc</i> 10: 49-64

Other biological activities that can be modulated by the HDAC genes and gene products are listed in the REFERENCE Table. Assays for detecting such biological activities are described in the Protein Domain table.

Profiles of these different HDAC genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up Regulated Transcripts	Responders To HDAC Activity	<ul style="list-style-type: none"> <li>• Gene Repression Activity</li> <li>• Cell Cycle Progression</li> <li>• Chromatin Condensation</li> <li>• Synthesis Of Metabolites And/Or Proteins</li> <li>• Modulation Of Transduction Pathways</li> <li>• Specific Gene Transcription Initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Transporters</li> <li>• Metabolic enzymes</li> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> </ul>
Down-Regulated Transcripts	Responder To Hdac Inhibitors  Genes With Discontinued Expression Or Unstable Mrna In	<ul style="list-style-type: none"> <li>• Negative Regulation Of Acetylation Pathways</li> <li>• Changes In Pathways And Processes</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or dephosphorylation (phosphatases)</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	Presence Of Hdac	<ul style="list-style-type: none"><li>Operating In Cells</li><li>• Changes In Metabolism</li></ul>	<ul style="list-style-type: none"><li>• Change in chromatin structure and/or DNA topology</li><li>• Stability of factors for protein synthesis and degradation</li><li>• Metabolic enzymes</li></ul>

#### USE OF PROMOTERS OF HISTONE DEACETYLASE RESPONSIVE GENES

Promoters of Histone Deacetylase responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Histone Deacetylase responsive genes where the desired sequence is operably linked to a promoter of a Histone Deacetylase responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.E. STRESS RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

#### **III.E.1. COLD RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

The ability to endure low temperatures and freezing is a major determinant of the geographical distribution and productivity of agricultural crops. Even in areas considered suitable for the cultivation of a given species or cultivar, can give rise to yield decreases and crop failures as a result of aberrant, freezing temperatures. Even modest increases (1-2°C) in the freezing tolerance of certain crop species would have a dramatic impact on agricultural productivity in some areas. The development of genotypes with increased freezing tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

Sudden cold temperatures result in modulation of many genes and gene products, including promoters. Examples of such cold responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix tables, MA\_diff and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to cold treatment.

Manipulation of one or more cold responsive gene activities is useful to modulate the biological activities and/or phenotypes listed below. Cold responsive genes and gene products can act alone or in combination. Useful combinations include cold responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of the levels of such proteins is especially useful for altering phenotypes and biochemical activities of plants. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108578, 108579, 108533, 108534). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown

for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Cold genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a "+" or "-" indication.

Cold Genes Identified By Cluster Analyses Of Differential Expression

Cold Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Cold genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108578, 108579, 108533, 108534 of the MA\_diff table(s).

Cold Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Cold genes. A group in the MA\_clust is considered a Cold pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Cold Genes Identified By Amino Acid Sequence Similarity

Cold genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Cold genes. Groups of Cold genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Cold pathway or network is a group of proteins that also exhibits Cold functions/utilities.

Such cold responsive genes and their products can function to either increase or dampen the phenotypes and activities below either in response to cold treatment or in the absence of cold temperature fluctuations.

Further, promoters of cold responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by ABA or any of the following phenotypes or biological activities below.

III.E.1.a. Use Of Cold-Responsive Genes To Modulate Phenotypes

Cold responsive genes and gene products are useful to or modulate one or more phenotypes including cold tolerance, below 7°C, for example, cells, organelles, proteins, dehydration resistance, growth rate, whole plant, including height, bolting time, etc., organs, biomass, fresh and dry weight during any time in plant life, such as maturation, number, size, and/or weight of flowers, seeds, branches, or leaves; seed yield in terms of number, size, weight, harvest index, or water content, fruit yield in terms of number, size, weight, harvest index, water content.

To regulate any of the phenotype(s) above, activities of one or more of the cold responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and assayed, for example, in accordance to Steponokus et al. (1993) Biochimica et Biophysica Acta 1145: 93-104; Quinn (1988) Symp Soc. Exp. Biol. 42: 237-258; Bectold and Pelletier (1998) Methods Mol. Biol. 82: 259-266; Kasuga et al. (1999) Nature Biotechnology 17: 287-291; Guy et al. (1998) Cryobiology 36: 301-314; or Liu et al. (1998) Plant Cell 10: 1391-1406.

III.E.1.b. Use Of Cold-Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the cold responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological

activities are documented and can be measured according to the citations above and those included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cold Tolerance	Viability Of Plant Protoplasts At Low Temperatures.	Steponkus (1998) PNAS USA 95: 14570-14575
	Viability Of Yeast At Low Temperatures.	Schirmer et al. (1994) Plant Cell 6: 1899-1909
	Complementation Of Yeast Tsp Mutant	Zentella et al. (1999) Plant Physiology, 119: 1473-1482
	Viability Of E.Coli At Low Temperatures.	Yeh et.al. (1997) PNAS 94: 10967-10972
	Induction Of Cold Shock Response Genes	Pearce (1999) Plant Growth Regulation 29: 47-76.
Lipid Composition	Altered Composition Of Membrane Fatty Acids	Sayanova et al. (1999) Journal of Experimental Botany 50: 1647-1652 Sayanova (1997) PNAS USA 94: 4211-4216
	ALTERATION OF LIPOXYGENASE ENZYME ACCUMULATION AND ACTIVITY	Porta et al. (1999) Plant and Cell Physiology 40: 850-858.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Protein Composition	<ul style="list-style-type: none"> <li>- PROTEIN DENATURATION</li> <li>- Protein Hydrophilicity</li> </ul>	<p>Wisniewski et al.(1999) <i>Physiologia Plantarum</i> 105: 600-608</p> <p>Steponkus (1998) <i>PNAS USA</i> 95: 14570-14575</p>
Modulation of Transcription Induced by Low Temperatures	<ul style="list-style-type: none"> <li>- Induced Transcription Factors And Other Dna Binding Proteins</li> <li>- Transcription Of Specific Genes</li> </ul>	<p><i>Current Protocols in Molecular Biology</i> / edited by Frederick M. Ausubel .. [et al.]. New York : Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, c1987.</p> <p>Steponkus (1998) <i>PNAS USA</i> 95: 14570-14575</p> <p>Kadyrzhanova et al., <i>Plant Mol Biol</i> (1998) 36(6): 885-895; and Pearce et al., <i>Plant Physiol</i> (1998) 117(3): 787-795</p>
Signal Transduction	<u>Plasma Membrane</u> <u>Proteins</u>	<p>Goodwin et al., <i>Plant Mol Biol</i> (1996) 31(4) 777-781; and</p> <p>Koike et al., <i>Plant Cell Physiol</i> (1997) 38(6): 707-716</p>
Oxygen Scavengers	<ul style="list-style-type: none"> <li>- Glutathione</li> <li>- Accumulation Active O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> Scavengers</li> </ul>	<p>Kocsy et al., <i>Planta</i> (2000) 210(2): 295-301</p> <p>Tao et al., <i>Cryobiology</i> (1998) 37(1):38-45</p>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Dehydration	<ul style="list-style-type: none"> <li>- Dehydrin</li> <li>- Transcription of mRNA</li> </ul>	<p>Ismail et al., Plant Physiol (1999)      120(1):237-244</p> <p>Kaye et al., Plant Physiol (1998)      116(4): 1367-1377</p>
Metabolism	Soluble Sugars and/or Proline	Wanner et al., (1999) Plant Physiol 120(2): 391-400
RNA/DNA Chaperone	Stabilization of RNA/DNA through RNA binding and modulation of RNA translation through RNA binding and or unwinding.	Jiang, Weining et al.,(1997) Journal of Biological Chemistry, 272: 196-202. Fukunaga et al., (1999) Journal of Plant Research, 112: 263-272.
Protein Chaperone	Stabilize protein structure and facilitate protein folding	<u>Forreiter and Nover (1998) Journal of Biosciences 23: 287-302</u>

Other biological activities that can be modulated by the cold responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Cold responsive genes are characteristically differentially expressed in response to fluctuating cold temperature levels, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various cold responsive genes in the aerial parts of seedlings at 1 and 6 hours at 4°C in the dark as compared to aerial parts of seedlings covered with aluminium foil, and grown at 20°C in the growth chamber.

The data from this time course can be used to identify a number of types of cold responsive genes and gene products, including “early responders” and “delayed responders”.

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Attorney Dkt. 2750-1571P  
Filed August 22, 2003

Profiles of these different cold responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Upregulated Genes (Level At 1 h $\geq$ 6 h) or (Level At 1 h > 6 h)	Early Responders To Cold	<ul style="list-style-type: none"> <li>- Perception Of Cold</li> <li>- Induction Of Cold Response Signal Transduction Pathway</li> <li>- Initiating Specific Gene Transcription</li> <li>- Osmotic Adjustment</li> <li>- Alteration Of Lipid Composition.</li> <li>- Ice Nucleation Inhibition</li> <li>- Mitigation Of Dehydration By Sequestering Water</li> </ul>	<ul style="list-style-type: none"> <li>- Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>- Amino Acid Sugar And Metabolite Transporters</li> <li>- Carbohydrate Catabolic And Anabolic Enzymes.</li> <li>- Lipid Biosynthesis Enzymes</li> <li>- Lipid Modification Enzymes, Example Desaturases</li> <li>- Ice Crystal Binding Proteins</li> <li>- Hydrophilic Proteins</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
	Stress Response	<ul style="list-style-type: none"> <li>- Repression Of General Biochemical Pathways To Optimize Cold Response Pathways.</li> <li>- Stabilization Of Protein /Enzyme Activity At Low Temperature</li> <li>- Protection Against Oxidative Stress</li> <li>-Anaerobic Metabolism</li> </ul>	<ul style="list-style-type: none"> <li>-Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>- Protein Stability Factors</li> <li>- mRNA Stability Factors</li> <li>- mRNA Translation Factors</li> <li>- Protein Turnover Factors</li> <li>Oxygen Radical Scavengers, Example- Peroxidases</li> <li>- Energy Generation Enzymes EtOH</li> <li>Detoxification</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Upregulated Genes (Level At 1h < 6 h)	Delayed Responders To Cold Stress - Cold Acclimation Genes	- Respiration, Photosynthesis And Protein Synthesis - Carbohydrate And Amino Acid Solute Accumulation - Increased Fatty Acid Desaturation To Increase Lipid Membrane Stability - Increased Accumulation Or Activity Of Oxidative Stress Protection Proteins - Stabilization Of Protein /Enzyme Activity At Low Temperature - Protection Against Oxidative Stress - Extracellular Matrix Modification	-Transcription Factors - Kinases And Phosphatases - Protein Stability Factors - mRNA Stability Factors - mRNA Translation Factors - Protein Turnover Factors - Oxygen Radical Scavengers, Peroxidase - Metabolic Enzymes

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
	Stress Response Genes	- Stabilization Of Protein /Enzyme Activity At Low Temperature - Protection Against Oxidative Stress - Anaerobic Metabolism	- Transcription Factors - Kinases And Phosphatases - Protein Stability Factors - mRNA Stability Factors - mRNA Translation Factors - Protein Turnover Factors - Oxygen Radical Scavengers, Example- Peroxidase - Energy Generation Enzymes, Etoh Detoxification

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Downregulated (Level At 1 h $\geq$ 6 h) (Level At 6 h > 1 h)	- Early Responder Repressors Of Cold Stress Metabolism  - Genes With Discontinued Expression Or UnsTable mRNA In Cold	- Negative Regulation Of Cold Signal Transduction Pathways Released  - Negative Regulation Of Cold Induced Transcription Reduced - Reduction In Gene Expression In Pathways Not Required Under Cold Conditions - Induced mRNA Turnover	- Transcription Factors - Kinases And Phosphatases - Protein Stability Factors - mRNA Stability Factors - mRNA Translation Factors - Protein Turnover Factors  - Cold Repressed Metabolic Pathway Proteins - Factors Coordinating And Controlling Central C and N Metabolism - Storage Proteins

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Down-Regulated Transcripts (Level At 1 h > 6 h)	<ul style="list-style-type: none"> <li>- Delayed Responder</li> <li>Repressors Of Cold Stress Metabolism</li> <li>- Genes With Discontinued Expression Or UnsTable mRNA In Cold</li> </ul>	<ul style="list-style-type: none"> <li>- Maintenance Of Cold Induced State Of Metabolism</li> <li>- Reduction In Gene Expression For Pathways Not Required Under Cold Conditions</li> <li>- Induced mRNA Turnover</li> </ul>	<ul style="list-style-type: none"> <li>Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>- Protein Stability Factors</li> <li>- mRNA Stability Factors</li> <li>- mRNA Translation Factors</li> <li>- Protein Turnover Factors</li> <li>- Cold Repressed Metabolic Pathway Proteins</li> <li>- Factors Coordinating And Controlling Central C and N Metabolism</li> <li>- Storage Proteins</li> </ul>

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the cold responsive genes when the desired sequence is operably linked to a promoter of a cold responsive gene.

### **III.E.2. HEAT RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

The ability to endure high temperatures is a major determinant of the geographical distribution and productivity of agricultural crops. Decreases in yield and crop failure frequently occur as a result of aberrant, hot conditions even in areas considered suitable for the cultivation

of a given species or cultivar. Only modest increases in the heat tolerance of crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased heat tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

Changes in temperature in the surrounding environment or in a plant microclimate results in modulation of many genes and gene products. Examples of such heat stress responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to high temperatures.

While heat stress responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different heat stress responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a heat stress responsive polynucleotide and/or gene product with other environmentally responsive polynucleotide is also useful because of the interactions that exist between stress pathways, pathogen stimulated pathways, hormone-regulated pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles, but which participate in common or overlapping pathways. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108576, 108577, 108522, 108523). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Heat genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Heat Genes Identified By Cluster Analyses Of Differential Expression

Heat Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Heat genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108576, 108577, 108522, 108523 of the MA\_diff table(s).

Heat Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Heat genes. A group in the MA\_clust is considered a Heat pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Heat Genes Identified By Amino Acid Sequence Similarity

Heat genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Heat genes. Groups of Heat genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Heat pathway or network is a group of proteins that also exhibits Heat functions/utilities.

Such heat stress responsive genes and gene products can function either to increase or dampen the above phenotypes or activities either in response to changes in temperature or in the absence of temperature fluctuations.

Further, promoters of heat responsive genes, as described in the Reference tables, for

example, are useful to modulate transcription that is induced by heat or any of the following phenotypes or biological activities below.

**III.E.2.a. Use Of Heat Stress Responsive Genes To Modulate Phenotypes**

Heat stress responsive genes and gene products can be used to alter or modulate one or more phenotypes including heat tolerance (above 20°C, 23°C, 27°C, 30°C, 33°C, 37°C, 40°C or 42°C), heat tolerance of of cells, of organelles, of proteins, of cells or organelles dehydration resistance, growth rate, whole plant, including height, bolting time, etc., organs, biomass, fresh and dry weight during any time in plant life, such as maturation, number, size, and weight of flowers, seeds, branches, or leaves; seed yield in number, size, weight, harvest index; fruit yield in terms of number, size, weight, or harvest index, stress responses such as mediation of response to desiccation, drought, salt, disease, wounding, cold and other stresses, and reproduction

To regulate any of the phenotype(s) above, activity of one or more of the heat stress responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Queitsch et al. (2000, The Plant Cell 12: 479-92).

**III.E.2.b. Use Of Heat Stress Responsive Genes To Modulate Biochemical Activities**

The activities of one or more of the heat stress responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
Cell Growth and Differentiation	<ul style="list-style-type: none"> <li>-Regulation And Molecular Chaperones</li> <li>-Maintenance Of Native Conformation (Cytosolic Proteins)</li> <li>-Reactivation Of Aggregation And Protein Folding</li> <li>-Autoregulation Of Heat Shock Response</li> <li>-Regulation Of Translational Efficiency</li> <li>-Regulation Of Kinase Activity</li> <li>-Regulation Of Calcium Mediated Signal Transduction</li> </ul>	<ul style="list-style-type: none"> <li>Wisniewski et al. (1999) Physiogia Plantarum 105: 600-608</li> <li>Queitsch et al. (2000) The Plant Cell 12: 479-92</li> <li>Lee and Vierling (2000) Plant Physiol. 122: 189-197</li> <li>Schwechheimer (1998) Plant Mol Biol 36: 195-204</li> <li>Shi et al. (1998) Genes and Development 12: 654-66</li> <li>Wells et al. (1998) Genes and Development 12: 3236-51</li> <li>Lis et al. (2000) Genes and Development 14: 792-803</li> </ul>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
		<p>-Malho, R.(1999) Plant Biology 1: 487-494.</p> <p>-Sheen, Jen.(1996) Science 274: 1900-1902.</p> <p>- Farmer, P. et al., (1999.) Biochimica et Biophysica Acta 1434: 6-17.</p>
Gene regulation	<ul style="list-style-type: none"><li>• Transcriptional Regulation Of Heat Induced Proteins Through DNA Binding Proteins.</li><li>• Transcriptional Regulation Of Heat Induced Proteins Through Protein-Protein</li></ul>	<p>-Current Protocols in Molecular Biology / edited by Frederick M. Ausubel .. [et al.]. New York : Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, c1987.</p> <p>-Steponkus (1998) PNAS USA 95: 14570-14575</p> <p>- Gubler et al. (1999) Plant Journal 17: 1-9</p> <p>- Glenn et al. (1999) Journal of</p>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
	<p>Interactions Between DNA Binding Proteins And Coactivators.</p> <ul style="list-style-type: none"> <li>Transcriptional Regulation Of Heat Induced Proteins Through Protein Phosphorylation And Dephosphorylation</li> <li>Transcriptional Regulation Of Thermal Stress Induced Genes By Protein-Protein Interactions.</li> <li>Translational Regulation Of Thermal Stress Induced Messenger Rnas.</li> <li>Transcriptional Regulation Of Heat Induced Genes Through Chromatin Remodeling.</li> </ul>	<p>Biological Chemistry, 274: 36159-36167</p> <p>- Zhou et al., (1997) EMBO Journal 16:3207-3218. - Sessa et al., (2000) EMBO Journal 19: 2257-2269. - Burnett et al.,(2000) Journal of Experimental Botany. 51: 197-205. - Osterlund et al.,(2000) Nature 405: 462-466. - Gross and Watson (1998) Canadian Journal of Microbiology,</p>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
		44:341-350  - Luo, R. X., Dean, D.C. (1999)  Journal of the National Cancer Institute 91: 1288- 1294.  -Chromatin protocols (1999) edited by Peter B. Becker. Totowa, N.J. : Humana Press.
Cell Structure	• Thermal Stress Protection By Plasma Membrane Anchored Or Secreted And/Or Cell Wall Associated Proteins.	- Goodwin et al. (1996) Plant Mol Biol 31(4) 777- 781; and Koike et al. (1997) Plant Cell Physiol 38(6): 707- 716
Signal Transduction	• Regulation Of Thermal Stress Pathways And Protein Activity By Protein Kinase And Protein Phosphatase Mediated Phosphorylation And Dephosphorylation Respectively.	- Jonak (1996) Proceedings of the National Academy of Sciences of the United States of America, 93: 11274-11279.  - Monroy et al., (1998) Analytical Biochemistry 265: 183-185.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
Photosynthesis	<ul style="list-style-type: none"><li>• Regulation Of Photoprotection And Repair Of Photosystem II</li></ul>	Schroda et al. (1999) The Plant Cell 11: 1165-178 Oh and Lee (1996) J Plant Biol. 39: 301-07
Stress Response	<ul style="list-style-type: none"><li>• Regulation Of Cytosol Peroxide Levels</li><li>• Regulation Of Heat Shock Factor Binding</li><li>• Regulation Of Protein Stability During Thermal Stress</li><li>• Nucleocytoplasmic Export Of Heat Shock Protein Mrnas</li><li>• Regulation/Reconfiguration Of Cell Architecture</li></ul>	Dat et al. (1998) Plant Physiol 116: 1351-1357  Kurek et al. (1999) Plant Physiol 119: 693-703  Storozhenko et al. (1998) Plant Physiol 118: 1005-14 Soto et al. (1999) Plant Physiol 120: 521-28 Yeh et al. (1997) PNAS 94: 10967-10972 Winkler et al. (1998) Plant Physiol 118: 743-50 Saavedra et al. (1997) Genes and Development 11: 2845-2856  Parsell and Lindquist (1993). Ann. Rev. Genet. 27: 437-496.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
	<ul style="list-style-type: none"><li>• Regulation Of Pathways For Reactivation Of "Damaged" And/Or Denatured Proteins</li><li>• Regulation Of Protein Degradation During Thermal Stress.</li><li>• Regulation Of Osmotic Potential During Thermal Stress.</li><li>• Regulation Of Universal Stress Protein Homologue Activity By Phosphorylation And Dephosphorylation.</li><li>• Regulation Of Dehydrin,</li></ul>	<p>Parsell and Lindquist (1993). <i>Ann. Rev. Genet.</i> 27: 437-496.</p> <p>Georgopoulos and Welch (1993). <i>Ann Rev. Cell Biol.</i> 9:601-634.</p> <p>- Vierstra, Richard D. (1996) <i>Plant Molecular Biology</i>, 32:275-302.</p> <p>- Vierstra, Richard D.; Callis, Judy. (1999) <i>Plant Molecular Biology</i>, 41:435-442.</p> <p>- Liu, J. et al., (1998) <i>Plant Science</i> 134:11-20.</p> <p>- Freestone, P. 1997 et al., <i>Journal of Molecular Biology</i>, v. 274: 318-324.</p> <p>- Robertson, A.J. (1994) <i>Plant Physiology</i> 105: 181-190.</p>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAY
	LEA-Like And Other Heat Stable Protein Accumulation	

Other biological activities that can be modulated by the heat stress responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Heat stress responsive genes are characteristically differentially transcribed in response to fluctuating temperatures, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various heat stress responsive genes in aerial tissues at 1 and 6 hours after plants were placed at 42°C as compared to aerial tissues kept at 20°C growth chamber temperature.

The data from this time course can be used to identify a number of types of heat stress responsive genes and gene products, including "early responders to heat stress," "delayed responders to heat stress," "early responder repressors," and "delayed repressor responders."

Profiles of these different heat stress responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES / GENE PRODUCTS
Up Regulated Transcripts (Level At 1h ≈ 6h) Or	Early Responders To Heat Stress	<ul style="list-style-type: none"> <li>• Heat Stress Perception</li> <li>• Modulation Of Heat Stress Response</li> <li>• Transduction</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Transporters</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES / GENE PRODUCTS
(Level At 1h > 6h)		<ul style="list-style-type: none"> <li>Pathways</li> <li>• Specific Gene Transcription Initiation</li> <li>• Conditional Shift In Preferential Translation Of Transcripts</li> <li>• Changes In Cell Architecture To Optimize Cell Adaptation To Heat Stress</li> </ul>	<ul style="list-style-type: none"> <li>• Changes In Cell Membrane Structure</li> <li>• Kinases And Phosphatases</li> <li>• Transcription Activators</li> <li>• Changes In Chromatin Structure And/Or Localized Dna Topology</li> <li>• Modification Of Pre-Existing Translation Factors By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li> <li>• Synthesis Of New Translation Factors</li> <li>• Stability Of Mediators Of Protein-Protein Interaction</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES / GENE PRODUCTS
			<p>Heat Shock Proteins          Changes In          Organelle          Structures,          Membranes And          Energy-Related          Activities</p> <p>Proteins To Catalyse          Metabolic Turnover</p>
Up Regulated Transcripts (Level At 1h < 6h)	<p>"Delayed" Responders</p> <p>Maintenance Of Heat Stress Response</p>	<ul style="list-style-type: none"> <li>• Maintenance Of Response To Heat Stress</li> <li>• Maintenance Of Protein Stability And Conformation</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Specific Factors (Initiation And Elongation) For Protein Synthesis</li> <li>• Maintenance Of Mrna Stability</li> <li>• Heat Shock Proteins</li> <li>• Changes In Organelle Structures,          Membranes And Energy-Related Activities</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES / GENE PRODUCTS
			<ul style="list-style-type: none"> <li>• Proteins To Catalyse Metabolic Turnover.</li> <li>• Stability Of Mediators Of Protein-Protein Interaction</li> </ul>
Down-Regulated Transcripts (Level At 1h ≈ 6h) Or (Level At 6h > 1h)	Early Responder Repressors Of "Normal" State Of Metabolism  Genes With Discontinued Expression Or Unstable mRNA In Presence Of Heat Stress	<ul style="list-style-type: none"> <li>• Negative Regulation Of Heat Stress Response Released</li> <li>• Changes In Biochemical And Signal Transduction Pathways And Processes Operating In Cells</li> <li>• Reorientation Of Metabolism</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors And Activators</li> <li>• Change In Protein Structure By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li> <li>• Change In Chromatin Structure And/Or Dna Topology</li> </ul>
Down-Regulated Transcripts (Level At 1hr > 6hr)	Delayed Repressors Of "Normal" State Of Metabolism  Genes With	<ul style="list-style-type: none"> <li>• Maintenance Of Heat Stress Response</li> <li>• Maintenance Of Pathways Released From Repression</li> <li>• Changes In Pathways</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors And Activators</li> <li>• Kinases And Phosphatases</li> <li>• Stability Of Factors</li> </ul>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES / GENE PRODUCTS
	Discontinued Expression Or Unstable mRNA In Presence Of Heat Stress	And Processes Operating In Cells • Reorientation Of Metabolism	For Protein Translation

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the heat responsive genes when the desired sequence is operably linked to a promoter of a heat responsive gene.

### **III.E.3. DROUGHT RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

The ability to endure drought conditions is a major determinant of the geographical distribution and productivity of agricultural crops. Decreases in yield and crop failure frequently occur as a result of aberrant, drought conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the drought tolerance of crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased drought tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the microclimate.

Drought conditions in the surrounding environment or within a plant, results in modulation of many genes and gene products. Examples of such drought responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to availability of water.

While drought responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different drought responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways, or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of the levels of such proteins is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a drought responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in a common pathway. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108572, 108573, 108502, 108503, 108504, 108556, 108482, 108483, 108473, 108474, 108477). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Drought genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a "+" or "--" indication.

#### Drought Genes Identified By Cluster Analyses Of Differential Expression

#### Drought Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Drought genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108572, 108573, 108502, 108503, 108504, 108556, 108482, 108483, 108473, 108474, 108477 of the MA\_diff table(s).

Drought Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Drought genes. A group in the MA\_clust is considered a Drought pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Drought Genes Identified By Amino Acid Sequence Similarity

Drought genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Drought genes. Groups of Drought genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Drought pathway or network is a group of proteins that also exhibits Drought functions/utilities.

Such drought responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to drought conditions or in the absence of drought conditions. Further, promoters of drought responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by drought or any of the following phenotypes or biological activities below.

More specifically, drought responsive genes and gene products are useful to or modulate one or more phenotypes including growth, roots, stems, buds, leaves, development, cell growth, leaves, fruit development, seed development, senescence, stress responses, and mediates response to desiccation, drought, salt and cold.

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the drought responsive genes when the desired sequence is operably linked to a promoter of a drought responsive gene.

To produce the desired phenotype(s) above, one or more of the drought response genes or gene products can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, *Methods. Mol. Biol.* 82:259-266) and/or screened for variants as in Winkler et al. (1998) *Plant Physiol* 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Ruzin (1999, In: *Plant Microtechnique and Microscopy*, Oxford University Press, London) and Khanna-Chopra et al. (1999, *BBRC* 255:324-7).

Alternatively, the activities of one or more of the drought responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	ASSAY
Cell Growth and Differentiation	Preservation of Leaf Sub-Cellular Structures Including Photosynthetic Apparatus  Preservation of Cell Membrane Structures  Regulation of Stomatal development and Physiology  Regulation of Factors Involved in the Drought-adapted change in cell ultrastructure	Jagtap et al. (1998) <i>J Exptl Botany</i> 49:1715-1721  Munne-Bosch and Alegre (2000) <i>Planta</i> 210: 925-31  Menke et al. (2000) <i>Plant Physiol.</i> 122:677-686.  Harrak et al. (1999) <i>Plant Physiol.</i> 121:557-564.

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	ASSAY
Physiology	Modulation of Transpiration	Allen et al. (1999) Plant Cell 11: 1785-98 Li et al. (2000) Science 287: 300-303 Burnett et al. (2000) J Exptl Bot 51: 197-205 Raschke (1987) In: Stomatal function, Zeiger et al., Eds, 253-79
	Modulation of Photosynthesis	Sung and Krieg (1979) Plant Physiol 64: 852-56
	Regulation of Epicuticular Wax Biosynthesis	Rhee et al. (1998) Plant Physiol 116: 901-11
	Regulation of Carotenoid Biosynthesis	Alegre (2000) Planta 210: 925-31 Loggini et al (2000) Plant Physiol 119:1091
Stress Response	Modulation of Leaf Rolling to minimize water loss	Taiz and Zeiger (1991) In: Plant Physiology, Benjamin/Cummings Publishing Co., Redwood City, pp 346-70
	Modulation of Osmolite	Hare et al. (1998) Plant,

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	ASSAY
	Synthesis  Regulation of gene transcriptional activity specific to the establishment of drought tolerance  Regulation of protein degradation and reactivation during drought stress condition  Modulation/reconfiguration of translation machineries ("recycling" mechanisms) adapTable to drought condition	Cell and Environment 21: 535-553  Huan et al. (2000) Plant Physiol 122: 747-756  Hare et al. (1999) J. Exptl. Botany 333:413-434.  Lee and Vierling (2000) Plant Physiol. 122: 189-197  Lis et al. (2000) Genes and Development 14: 792-803
Signal Transduction	Regulation of Ion Sequestration  Regulation of Nuclear Targeted Protein Transport  Regulation of Cytoplasmic Ca+2	Bush and Jones (1987) Cell Calcium 8: 455-72  Ferringno and Silver (1999) Methods in Cell Biology 58: 107-22

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	ASSAY
	Regulation of Kinase Synthesis and Activity  Modulation of Molecular Chaperone Activity	Shi et al. (1999) Plant Cell 11: 2393-2406  Li et al. (2000) Science 287- 300-03  Mayhew et al (1996) Nature 379: 420-26 Kimura et al. (1995) Science 268:1362-1365.

Other biological activities that can be modulated by the drought responsive genes and gene products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Protein Domain table.

Drought responsive genes are characteristically differentially transcribed in response to drought conditions, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various drought responsive genes at 1 and 6 hours after aerial tissues were isolated and left uncovered at room temperature on 3 MM paper, as compared to isolated aerial tissues placed on 3 MM paper wetted with Hoagland's solution. The data from this time course can be used to identify a number of types of drought responsive genes and gene products, including "early responders," and "delayed responders." Profiles of these different drought responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Up regulated transcripts  (level at 1 hr ≈ 6 hr)  (level at 1 hr > 6 hr)	Early responders to drought	Drought perception leading to the establishment of tolerance to drought  Modulation of drought response transduction pathways  Specific gene transcription initiation  Conditional shift in preferential translation of transcripts  Changes in cell architecture to optimize cell adaptation to heat	Transcription factors  Transporters   Change in cell membrane structure  Kinases and phosphatases   Transcription activators  Change in chromatin structure and/or localized DNA topology   Modification of pre-existing translation factors by phosphorylation (kinases) or dephosphorylation (phosphatases)  Synthesis of new translation factors   Stability of mediators of protein-protein interaction

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
		<p>stress</p> <p>Changes in cell division cycle</p>	<p>Synthesis and/or stability of factors regulating cell division</p>
Up regulated transcripts (level at 1 hr < 6 hr)	<p>Maintenance of drought response</p> <p>"Delayed" responders</p>	<p>Maintenance of response to drought and maintenance of drought-tolerance mechanisms</p> <p>Maintenance of mechanisms effective for ions sequestration, osmolite biosynthesis, nuclear protein transport, regulation of cytoplasmic Ca+2, and regulation of proteins effective for</p>	<p>Transcription factors</p> <p>Specific factors (initiation and elongation) for protein synthesis</p> <p>RNA-binding proteins effective for mRNA stability</p> <p>Change in chromatin structure and/or DNA topology</p> <p>Stability of mediators of protein-protein interaction</p> <p>Stability of factors to effectively utilize pre-existing translation machinery ("recycling" mechanisms) under drought condition</p>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
		<p>maintaining protein stability and conformation</p> <p>Maintenance of cellular structures</p>	<p>Stability of mediators of protein-protein interaction</p>
Down-regulated transcripts (level at 1 hr ≈ 6 hr) (level at 6 hr > 1 hr)	<p>Early responder repressors of "normal" state of metabolism</p> <p>Genes with discontinued expression or unsTable mRNA in presence of water stress</p>	<p>Negative regulation of drought response inducible pathways released</p> <p>Changes in biochemical and signal transduction pathways and processes operating in cells</p>	<p>Transcription factors and activators</p> <p>Change in protein structure by phosphorylation (kinases) or dephosphoryaltion (phosphatases)</p> <p>Change in chromatin structure and/or DNA topology</p>
Down-regulated transcripts (level at 1 hr > 6 hr)	<p>Delayed repressors of "normal" state of metabolism</p> <p>Genes with discontinued expression or unsTable mRNA in</p>	<p>Maintenance of drought response</p> <p>Maintenance of pathways released from repression</p> <p>Changes in pathways and processes operating in cells</p>	<p>Transcription factors and activators</p> <p>Kinases and phosphatases</p> <p>Stability of factors for protein translation</p>

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
	presence of water stress		

#### USE OF PROMOTERS OF DROUGHT RESPONSIVE GENES

Promoters of Drought responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Drought responsive genes where the desired sequence is operably linked to a promoter of a Drought responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.E.4. WOUNDING RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Plants are continuously subjected to various forms of wounding from physical attacks including the damage created by pathogens and pests, wind, and contact with other objects. Therefore, survival and agricultural yields depend on constraining the damage created by the wounding process and inducing defense mechanisms against future damage.

Plants have evolved complex systems to minimize and/or repair local damage and to minimize subsequent attacks by pathogens or pests or their effects. These involve stimulation of cell division and cell elongation to repair tissues, induction of programmed cell death to isolate the damage caused mechanically and by invading pests and pathogens, and induction of long-range signaling systems to induce protecting molecules, in case of future attack. The genetic and biochemical systems associated with responses to wounding are connected with those associated with other stresses such as pathogen attack and drought.

Wounding results in the modulation of activities of specific genes and, in consequence, of the levels of key proteins and metabolites. These genes, called here wounding responsive genes, are important for minimizing the damage induced by wounding from pests, pathogens and other objects. Examples of such wounding responsive genes, gene components and products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff, and MA\_clust tables. They can be active in all parts of a plant and so where, when and to what extent they are active is crucial for agricultural performance and for the quality, visual and otherwise, of harvested products. They were discovered and characterized from a much larger set of genes by experiments designed to find genes whose products changed in response to wounding.

Manipulation of one or more wounding responsive gene activities is useful to modulate the biological activities and/or phenotypes listed below. Wounding responsive genes and gene products can act alone or in combination with genes induced in other ways. Useful combinations include wounding responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of the levels of such proteins is especially useful for altering phenotypes and biochemical activities of plants. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108574, 108575, 108524, 108525, and Wounding (relating to SMD 3714, SMD 3715)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Wounding genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Wounding Genes Identified By Cluster Analyses Of Differential Expression

#### Wounding Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the

microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Wounding genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108574, 108575, 108524, 108525, and Wounding (relating to SMD 3714, SMD 3715) of the MA\_diff table(s).

Wounding Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Wounding genes. A group in the MA\_clust is considered a Wounding pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Wounding Genes Identified By Amino Acid Sequence Similarity

Wounding genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Wounding genes. Groups of Wounding genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Wounding pathway or network is a group of proteins that also exhibits Wounding functions/utilities.

Such wounding responsive genes and gene products can function either to increase or dampen the phenotypes and activities below, either in response to wounding or in the absence of wounding.

Further, promoters of wounding responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by wounding or any of the following phenotypes or biological activities below.

III.E.4.a. Use Of Wounding-Responsive Genes To Modulate Phenotypes

Wounding responsive genes and gene products can be used to alter or modulate one or more phenotypes including growth rate; whole plant height, width, or flowering time; organs (such as coleoptile elongation, young leaves, roots, lateral roots, tuber formation, flowers, fruit, and seeds); biomass; fresh and dry weight during any time in plant life, such as at maturation; number of flowers; number of seeds; seed yield, number, size, weight, harvest index (such as content and composition, e.g., amino acid, nitrogen, oil, protein, and carbohydrate); fruit yield, number, size, weight, harvest index, post harvest quality, content and composition (e.g., amino acid, carotenoid, jasmonate, protein, and starch); seed and fruit development; germination of dormant and non-dormant seeds; seed viability, seed reserve mobilization, fruit ripening, initiation of the reproductive cycle from a vegetative state, flower development time, insect attraction for fertilization, time to fruit maturity, senescence; fruits, fruit drop; leaves; stress and disease responses; drought; heat and cold; wounding by any source, including wind, objects, pests and pathogens; uv and high light damage (insect, fungus, virus, worm, nematode damage).

To regulate any of the phenotype(s) above, activities of one or more of the wounding responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and assayed, for example, in accordance with Johnson et.al. (1998) Plant Physiol 116:643-649, Reymond et.al. (2000) Plant Cell 12 707-720, or Keith et.al. (1991) Proc. Nat. Acad. Sci.USA 888821 8825.

III.E.4.b. Use Of Wounding-Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the wounding responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations included in the Table below:

PROCESS	BIOLOGICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
<u>Plant Tissue Proliferation</u>	Cell Damage Repair; Cell Division	Flanders (1990) J. Cell Biol. 110: 1111-1122
Wound Induced Pathways Providing Defense Against Pests And Pathogens	Synthesis Of Jasmonic And Salicylic Acids And The Pathways Induced By These Signaling Molecules. Induction Of Jasmonic Acid Independent Defense Pathways. Induction Of Lipoxygenase, Thionins And Nodulins	Reymond, P and Farmer E.E. Current Opinion in Plant Biology 1998 1:404-411 Creelman, RA and Mullet, J.E. (1997) Ann Rev. Plant Physiol Mol Biol 48: 355-387 Leon et al. 1998 Mol Gen Genet 254: 412-419 Titarenko et al. 1997 Plant Physiol 115: 817-826
	Cell Wall Degradation, Ethylene Formation, Systemic Signaling And Induction Of Defense Related Genes	Rojo, E. et al. 1998. Plant J 13:153-165 Ryan, CA and Pearce, G. 1998. Ann Rev. Cell Dev. Biol 14: 1-17
	Specific Rnase Induction	Reymond, P. et al. 2000 . Plant Cell 12:707-720 Glazebrook, J. 1999. Current Opinion in Plant Biol. 2: 280-286 O'Donnell P. J., et al. 1996 Science 274: 1914-1917 Rojo et al. 1999. Plant J. 20: 135-142 Merkouropoulos G. et al.

PROCESS	BIOLOGICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
		1999 Planta 208: 212-219 Kariu et al. 1998. Bioscience Biotechnology and Biochemistry 62: 1144-1151 McOann et al. 1997 PNAS 94: 5473-5477
Other Stress Induced Pathways	Abscisic Acid Formation And Its Signaling Pathway Cold Responsive Genes and Pathways Drought Induced Dehydrins And Pathways	Carrera, E and Prat, S. 1998. Plant J 15: 767-771 Chao et. al. 1999. Plant Physiol 120: 979-992
Modified Lipid Metabolism	Membrane Lipid Synthesis Including Omega-3 Fatty Acid Desaturase Lipases Lipid Transfer Proteins	Martin, M et al. 1999 Europe J. Biochem 262: 283-290
Modified Sugar And Energy Metabolism	Induction Of Glycohydrolases And Glycotransferases, Amylases Induction Of Aminotransferases, Arginase, Proteases And Vegetative Storage Proteins, Aromatic Amino Acid Synthesis	
Modified Protein And Nitrogen Metabolism		

PROCESS	BIOLOGICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Secondary Metabolite Induction	Aromatic Amino Acid Synthesis And Secondary Metabolites	Keith, B et al. 1991 PNAS 88: 8821-8825

Other biological activities that can be modulated by wound responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

The MA\_diff table reports the changes in transcript levels of various wound responsive genes in the aerial parts of a plant, 1 and 6 hours after the plants were wounded with forceps. The comparison was made with aerial tissues from unwounded plants.

The data from this time course reveal a number of types of wound responsive genes and gene products, including "early responders," and "delayed responders." Profiles of the individual wounding responsive genes are shown in the Table below together with examples of the kinds of associated biological activities that are modulated when the activities of one or more such genes vary in plants.

TRANSCRIPT LEVELS	TYPES OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY

TRANSCRIPT LEVELS	TYPES OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up Regulated Transcripts  (Level At 1h ≈ 6h)  Or  (Level At 1h > 6h)	Early Responders To Wounding	<ul style="list-style-type: none"> <li>• Induction Of Key Signaling Pathways Within And Between Cells</li> <li>• Modulation Of Wounding And Stress Induced Signal Transduction Pathways</li> <li>• Specific Gene Transcription Initiation</li> <li>• Induction Of Repair Processes Or Cell Death</li> <li>• Reorientation Of Metabolism, Including Management Of Active Oxygen</li> <li>• Movement Of Wound</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Kinases And Phosphatases</li> <li>• Jasmonic Acid, Salicylic Acid And Nitric Oxide Pathway Proteins.</li> <li>• Glycohydrolases</li> <li>• Dehydrins</li> <li>• Rnases</li> <li>• Metabolic Enzymes</li> <li>• Nodulins</li> <li>• Cell Division And Cell Wall Proteins</li> <li>• Cold Response Proteins</li> <li>• Lipoxygenase</li> <li>• Jacalin</li> <li>• Proteins To Detoxify Active Oxygen Species</li> <li>• Systemin</li> </ul>

TRANSCRIPT LEVELS	TYPES OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>• Induced Signals Through Plant</li> <li>• Synthesis Of Phytoalexins And Secondary Metabolites</li> </ul>	<ul style="list-style-type: none"> <li>• Biosynthetic Enzymes</li> </ul>
Up Related Transcripts  ( Level At 1h < 6h)	<p>Delayed Responders</p> <p>Genes Involved In Wounding Response At Distant Sites From Wound.</p> <p>Genes Involved In Maintenance Of Wounding Response</p>	<ul style="list-style-type: none"> <li>• Maintenance Of Defence Pathways</li> <li>• Maintenance Of Reorientated Metabolism</li> <li>• Maintenance Of Wound Response</li> <li>• Programmed Cell Death In Selected Cells</li> <li>• Reorientation Of Metabolism</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Kinases And Phosphatases</li> <li>• Jasmonic Acid, Salicylic Acid And Nitric Oxide Pathway Proteins</li> <li>• Glycohydrolases</li> <li>• Dehydrins</li> <li>• Rnases</li> <li>• Metabolic Enzymes</li> <li>• Nodulins</li> <li>• Cold Response Proteins</li> </ul>

TRANSCRIPT LEVELS	TYPES OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>• Movement Of Wound Induced Signals Through Plant</li> <li>• Synthesis Of Phytoalexins And Secondary Metabolites</li> </ul>	<ul style="list-style-type: none"> <li>• Lipoxygenase</li> <li>• Jacalin</li> <li>• Proteins To Detoxify Active Oxygen Species</li> <li>• Cell Division And Cell Wall Proteins</li> <li>• Systemin</li> <li>• Biosynthetic Enzymes</li> </ul>
Down – Regulated Transcripts (Level At 1h ≈ 6h) Or (Level At 6 Hr > 1h)	<ul style="list-style-type: none"> <li>• Early Responder Repressors Of Wounding Response State</li> <li>• Genes With Discontinued</li> </ul>	<ul style="list-style-type: none"> <li>• Negative Regulation Of Wounding Response Pathways Released</li> <li>• Changes In Pathways And Processes Operating In Cells</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Change In Protein Structure By Phosphory-Lation (Kinases) Or Dephos-Phorylation (Phosphatases)</li> <li>• Change In Chromatin Structure And Or Dna Topology</li> <li>• Local Changes In Regulatory Proteins, Metabolic Enzymes,</li> </ul>

TRANSCRIPT LEVELS	TYPES OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	Expression Or Unstable mRNA Following Wounding		Transporters Etc.
Down – Regulated Transcripts (Level At 1hr > 6h)	Delayed Repressors Of Wounding Response State  Genes With Discontinued Expression Or Unstable mRNA Following Wounding	<ul style="list-style-type: none"> <li>• Negative Regulation Of Wounding Response Pathways Released</li> <li>• Change In Pathways And Process Operating In Cells</li> <li>• Programmed Cell Death</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors, Phosphatases, Kinases</li> <li>• Changes In Protein Complex Structures</li> <li>• Chromatin Restructuring Proteins</li> <li>• Local Changes In Regulatory Proteins, Metabolic Enzymes, Transporters Etc.</li> <li>• Most Proteins In Selected Cells Undergoing Death</li> </ul>

Further, any desired sequence can be transcribed in similar temporal, tissue, or

environmentally specific patterns as the wounding responsive genes when the desired sequence is operably linked to a promoter of a wounding responsive gene.

### **III.E.5. METHYL JASMONATE (JASMONATE) RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Jasmonic acid and its derivatives, collectively referred to as jasmonates, are naturally occurring derivatives of plant lipids. These substances are synthesized from linolenic acid in a lipoxygenase-dependent biosynthetic pathway. Jasmonates are signalling molecules which have been shown to be growth regulators as well as regulators of defense and stress responses. As such, jasmonates represent a separate class of plant hormones.

Changes in external or internal jasmonate concentration result in modulation of the activities of many genes and gene products. Examples of such "jasmonate responsive" genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield, especially when plants are growing in the presence of biotic or abiotic stresses. They were discovered and characterized from a much larger set of genes by experiments designed to find genes whose mRNA products changed in concentration in response to application of methyl jasmonate to plants.

Manipulation of one or more jasmonate responsive gene activities is useful to modulate the biological activities and/or phenotypes tested below. Jasmonate response genes and gene products can act alone or in combination. Useful combinations include jasmonate responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same co-regulated or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities. Such jasmonate responsive genes and gene products can function to either increase or dampen the phenotypes or activities below either in response to changes in jasmonate concentration or in the absence of jasmonate fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108568, 108569, 108555). For transcripts that had higher levels in the samples than the control, a "+" is shown. A "-" is shown for when transcript levels were

reduced in root tips as compared to the control. For more experimental detail see the Example section below.

MeJA genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

MeJA Genes Identified By Cluster Analyses Of Differential Expression

MeJA Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of MeJA genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108568, 108569, 108555 of the MA\_diff table(s).

MeJA Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of MeJA genes. A group in the MA\_clust is considered a MeJA pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

MeJA Genes Identified By Amino Acid Sequence Similarity

MeJA genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis MeJA genes. Groups of MeJA genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a MeJA pathway or network is a group of proteins that also exhibits MeJA functions/utilities.

Further, promoters of jasmonate responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by jasmonate or any of the following phenotypes or biological activities below.

III.E.5.a. Use Of Jasmonate Responsive Genes To Modulate Phenotypes:

Jasmonate responsive genes and their gene products can be used to alter or modulate one or more phenotypes including growth rate, whole plant (including height, flowering time, etc.), seedling, organ, coleoptile elongation, young leaves, roots, lateral roots, tuber formation, flowers, fruit, seeds, biomass; fresh and dry weight during any time in plant life, including maturation and senescence; number of flowers, number of seeds (including secondary metabolite accumulation, alkaloids, anthocyanins; paclitaxel and related taxanes, rosmarinic; seed yield (such as number, size, weight, harvest index, content and composition, e.g., amino acid, jasmonate, oil, protein, and starch); fruit yield (such as number, size, weight, harvest index, post harvest quality, content and composition e.g., amino acid, carotenoid, jasmonate, protein, starch); seed and fruit development; germination of dormant and non-dormant seeds; seed viability; seed reserve mobilization; fruit ripening (such as initiation of the reproductive cycle from a vegetative state); flower development time; insect attraction for fertilization; time to fruit maturity; senescence; fruits, fruit drop; leaves; stress and disease responses; drought; wounding; UV damage; and insect, fungus, virus, or worm damage.

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the jasmonate responsive genes when the desired sequence is operably linked to a promoter of a jasmonate responsive gene.

To improve any of the phenotype(s) above, activities of one or more of the jasmonate responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed, for example, in accordance to citations described below.

III.E.5.b. Use Of Jasmonate-Responsive Genes To Modulate Biochemical Activities:

The activities of one or more of the jasmonate responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Turnover of proteins	- Induction of various proteases, ubiquitin and proteosome components and turnover of RNA polymerases and translation initiation factors - Reduction in many ribosomal proteins	This study. Standard biochemical assays.
Activation of nitrogen metabolism	Induction of glutamine synthetase, many aminotransferases, vegetative storage proteins	Crawford (1995) Plant Cell 7, 859-868 This study. Standard biochemical assays.
Lipid turnover	Induction of various lipases, desaturases, and reduction of lipid transfer protein mRNAs	This study. Standard biochemical assays.
Sugar metabolism	Induction of sugar transporters, UDP glucosyltransferases, other transferases	This study. Standard biochemical assays.

Glycolysis and central carbon metabolism	Induction of glycolytic related enzymes . Example, glucose 6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglucomutase ATP synthase	This study. Standard biochemical assays.
Chlorosis	Degradation of Chlorophyll	Tsuchiya et al. (1999) Proc. Natl. Acad. Sci. U SA 96:15362-15367
	Inhibition of Photosynthesis Related Proteins	Reinbothe et al. (1993) J. Biol. Chem. 268, 10606-10611
Carbon Assimilation and turnover	Induction of chlorophyll ab binding protein precursor	Reinbothe et al. (1993) J. Biol. Chem. 268, 10606-10611
Jasmonate metabolism	Induction of lipid biosynthesis, myrosinase and jacalin	This study. Standard biochemical assays.
Jasmonate mediated signal transduction	Receptor binding	Cho and Pai (2000) Mol Cells 10, 317-324

	Protein kinases	Lee et al. (1998) Mol. Gen. Genet. 259, 516-522  Seo et al. (1999) Plant Cell 11, 289-298  Yoon et al. (1999) Plant Mol. Biol. 39, 991-1001
	Ubiquitination of Repressor Proteins	Xie et al. (1998) Science 280, 1091-1094
	Calcium Flux regulators	Bergey and Ryan (1999) Plant Mol. Biol. 40, 815-823
	Transcription Activators. Example- induction of various zinc finger, myb and AP-2 related factors	Xiang et al. (1996) Plant Mol. Biol. 32, 415-426  Menke et al. (1999) EMBO J. 18, 4455-4463
Response to Cell Membrane Damage	Lipid Peroxidation	Dubery et al. (2000) Mol. Cell Biol. Res. Commun. 3, 105-110
Cell Elongation	Inhibition of incorporation of Glucose into Cell Wall Saccharides	Burnett et al. (1993) Plant Physiol. 103, 41-48

Cell Organization and Division	- Reductions in tropomyosin related proteins and certain cyclins - Induction of actins and tubulins	Ishikawa et al. (1994) Plant Mol. Biol. 26, 403-414
Cell Wall Turnover and modulation	- Induction of cell wall proteins, glycine-rich proteins, annexins, pectate lyase and pectin esterases - Reductions in various dehydrins and expansins	Creelman et al. (1992) Proc. Natl. Acad. Sci. USA 89, 4938-4941  Garcia-Muniz et al. (1998) Plant Mol. Biol. 38, 623-632  Norman et al (1999) Mol. Plant Microbe Interact. 12, 640-644

Stress, Disease, and Pathogen Resistance	Induction of antifungal proteins, wounding responsive proteins, dehydrins, heat shock type proteins and elicitor response proteins	Hildmann et al. (1992) Plant Cell 4, 1157-1170  Reinbothe et al. (1994) Proc. Natl. Acad. Sci. USA 91, 7012-7016  Moons et al. (1997) Plant Cell 9, 2243-2259  Richard et al. (2000) Plant Mol. Biol. 43, 1-10  Van Wees et al. (2000) Proc. Natl. Acad. Sci. USA 97, 8711-8716
	Phytoalexin Biosynthesis	Creelman et al. (1992) Proc. Natl. Acad. Sci. USA 89, 4938-4941  Choi et al. (1994) Proc. Natl. Acad. Sci. USA 91, 2329-2333
	Biosynthesis of phenolics	Doares et al., (1995) Proc. Natl. Acad. Sci. USA 92, 4095-5098
	Production of Protease Inhibitors	Botella et al. (1996) Plant Physiol 112, 1201-1210

	Defense Gene Transcription in Response to UV	Mason et al. (1993) Plant Cell 5, 241-251  Schaller et al. (2000) Planta 210, 979-984
Secondary Metabolite biosynthesis	Fruit Cartenoid Composition	Czapski and Saniewski (1992) J. Plant Physiol. 139, 265-268
	Palitaxel and Related Taxanes	Yukimune et al. (1996) Nature Biotech. 14, 1129- 1132
	Alkaloids	Aerts et al. (1994) Plant J. 4, 635-643 Geerlings et al. (2000) J. Biol. Chem. 275, 3051-3056
	Anthocyanins	Franceschi et al. (1991) Proc. Natl. Acad. Sci. USA 83, 6745-6749
	Rosmarinic	Mizukami et al., (1993) Plant Cell Reprod. 12, 706-709
	Activation of Ethylene- forming Enzyme and Production of Ethylene	Czapski and Saniewski (1992) J. Plant Physiol. 139, 265-268

Other biological activities that can be modulated by the jasmonate responsive genes and their products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Domain section of the Reference Tables.

Jasmonate responsive genes are characteristically differentially transcribed in response to fluctuating jasmonate levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various jasmonate responsive genes in the aerial parts of a seedling at 1 and 6 hours after being sprayed with Silwet L-77 solution enriched with methyl jasmonate as compared to seedlings sprayed with Silwet L-77 alone.

The data from this time course reveal a number of types of jasmonate responsive genes and gene products, including "early responders" and "delayed responders". Profiles of the individual kinds of jasmonate responsive genes are shown in the Table below, together with examples of the kinds of associated biological activities that are modulated when the activities of such genes vary.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
Upregulated genes  (Level at 1 hour ≥ 6 hours).  (Level at 1 hour > 6 hours)	Early Responders to Jasmonate	Binding and Perception of Jasmonate  Transduction of Jasmonate signal transduction response pathways  Initiation of Specific Gene Transcription to reorientate metabolism	Transcription Factors Transporters  Kinases, Phosphatases, Leucine-rich Repeat Proteins (LRRs), GTP-binding proteins (G-proteins), calcium-binding proteins and calcium responsive proteins  Proteases, lipases, glutamine synthetase (GS), arginase, aminotransferases,

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
			glycosyltransferases, sugar transporters, cell wall proteins, methyl transferases, glycolytic enzymes.
Upregulated genes (Level at 1 hour < 6 hours)	Delayed Jasmonate Responders	Maintenance of Metabolism under high Jasmonate  Jasmonate signal  Tranduction Response Pathways  Gene Transcription to Reorientate  Metabolism	Enzymes of methyl jasmonate-induced pathways, including dehydrin, phytoalexin, phenolic, carotenoid, alkaloid and anthocyanin biosynthesis.  Transcription factors, Transporters, Kinases and phosphatases  Proteases, Lipases, Glutaminae  Synthetase, Arginase, Aminotransferases, Lipid Peroxidases, Glycosyltransferases, Sugar transporters, Cell Wall Proteins,

	EXAMPLES OF BIOCHEMICAL ACTIVITY
to ited	Glycolytic Enzymes, Chlorophyll Binding Proteins
	Transcription factors, kinases, phosphatases, LRRs, G-proteins
	Actins, Tubulins, Myosins Cyclins, Cyclin-dependent Kinases (CDPKs)
	Glycosyl Transferases, Glycosyl hydrolases, Expansins, Extensins, O- Methyl Transferases
	Arabinogalactan- proteins (AGPs), Enzymes of Lipid Biosynthesis, Cutinase.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	TYPE OF BIOLOGICAL ACTIVITY	EXAMPLES OF BIOCHEMICAL ACTIVITY
Down regulated transcripts  (level at 1 hour = 6 hours)  ( level at 6 hours >1 hour)	Early responders of Jasmonate  Genes with discontinued expression or unsTable mRNA following Jasmonate uptake	Release of Suppression of Jasmonate Induced Pathways  Reorientation of metabolism	Transcription Factors, Kinases, Phosphatases, LRRs, G-Proteins, Chromatin  Restructuring proteins, Ribosomal proteins, Translation Factors, Histones, RNA polymerases, Pectin esterase, Lipid transfer proteins
Down regulated transcripts  (level at 1 hour > 6 hours)	Genes with Discontinued expression or UnsTable mRNA Following Jasmonate uptake	Negative Regulation of Jasmonate Induced Pathways Released.  Reorientation of metabolism	Transcription factors Kinases, Phosphatases Chromatin Restructuring Proteins, LRRs, G-proteins  Ribosomal proteins, Translation Factors, Histones RNA Polymerases, Cyclins Pectin esterase, Lipid Transfer Proteins

### USE OF PROMOTERS OF JASMONATE RESPONSIVE GENES

Promoters of Jasmonate responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Jasmonate responsive genes where the desired sequence is operably linked to a promoter of a Jasmonate responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.E.6. REACTIVE OXYGEN RESPONSIVE GENES, GENE COMPONENTS AND H<sub>2</sub>O<sub>2</sub> PRODUCTS**

Often growth and yield are limited by the ability of a plant to tolerate stress conditions, including pathogen attack, wounding, extreme temperatures, and various other factors. To combat such conditions, plant cells deploy a battery of inducible defense responses, including triggering an oxidative burst. The burst of reactive oxygen intermediates occurs in time, place and strength to suggest it plays a key role in either pathogen elimination and/or subsequent signaling of downstream defense functions. For example, H<sub>2</sub>O<sub>2</sub> can play a key role in the pathogen resistance response, including initiating the hypersensitive response (HR). HR is correlated with the onset of systemic acquired resistance (SAR) to secondary infection in distal tissues and organs.

Changes in reactive oxygen, such as H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub><sup>-</sup>, in the surrounding environment or in contact with a plant results in modulation of the activities of many genes and hence levels of gene products. Examples of such reactive oxygen responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. The genes were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to application of reactive oxygen, such as H<sub>2</sub>O<sub>2</sub>, to plants.

Manipulation of one or more reactive oxygen responsive gene activities is useful to modulate the following biological activities and/or phenotypes listed below. Reactive oxygen

responsive genes and gene products can act alone or in combination. Useful combinations include reactive oxygen responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants.

Such reactive oxygen responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in reactive oxygen concentration or in the absence of reactive oxygen fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108582, 108583, 108537, 108538, 108558, and H2O2 (relating to SMD 7523)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Reactive Oxygen genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Reactive Oxygen Genes Identified By Cluster Analyses Of Differential Expression

#### Reactive Oxygen Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Reactive Oxygen genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108582, 108583, 108537, 108538, 108558, and H2O2 (relating to SMD 7523) of the MA\_diff table(s).

Reactive Oxygen Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Reactive Oxygen genes. A group in the MA\_clust is considered a Reactive Oxygen pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Reactive Oxygen Genes Identified By Amino Acid Sequence Similarity

Reactive Oxygen genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Reactive Oxygen genes. Groups of Reactive Oxygen genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Reactive Oxygen pathway or network is a group of proteins that also exhibits Reactive Oxygen functions/utilities.

Further, promoters of reactive oxygen responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by reactive oxygen or any of the following phenotypes or biological activities below.

III.E.6.a. Use Of Reactive Oxygen Responsive Genes To Modulate Phenotypes

Reactive oxygen responsive genes and gene products are useful to or modulate one or more phenotypes including pathogen tolerance and/or resistance; Avr/R locus sensitive; non-host sensitive; HR; SAR (e.g., where the reactive oxygen responsive gene and products are modulated in conjunction with any of the bacterial, fungal, virus, or other organism listed below); bacteria resistance, e.g. to *Erwinia stewartii*, *Pseudomonas syringae*, *Pseudomonas tabaci*, Stuart's wilt, etc.; fungal resistance including to downy mildews such as *Sclerotinia macrospora*, *Sclerotinia rayissiae*, *Sclerospora graminicola*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora sacchari*, *Peronosclerospora maydis*; rusts such as *Puccinia*

sorpii, Puccinia polyspora, Physopella zae, etc.; other fungal diseases such as Cercospora zae-maydis, Colletotrichum graminicola, Fusarium moniliforme, Exserohilum turcicum, Bipolaris maydis, Phytophthora parasitica, Peronospora tabacina, Septoria, etc.; virus or viroid resistance, e.g. to tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato bushy stunt virus, and like viruses; insect resistance, such as to aphids e.g. *Myzus persicae*; beetles, beetle larvae; etc.; nematodes, e.g. *Meloidogyne incognita*; lepidoptera, e.g. *Heliothis spp.* etc.; resistance specifically in primary or secondary leaves; stress tolerance; winter survival; cold tolerance; heavy metal tolerance, such as cadmium; physical wounding; increased organelle tolerance to redox stress, such as in mitochondria, and chloroplasts; cell death; apoptosis, including death of diseased tissue; senescence; fruit drop; biomass; fresh and dry weight during any time in plant life, such as maturation; number of flowers, seeds, branches, and/or leaves; seed yield, including number, size, weight, and/or harvest index; fruit yield, including number, size, weight, and/or harvest index; plant development; time to fruit maturity; cell wall strengthening and reinforcement; plant product quality, e.g. paper making quality); food additives; treatment of indications modulated by free radicals, and cancer

To regulate any of the phenotype(s) above, activities of one or more of the reactive oxygen responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and assayed , for example, in accordance to Alvarez et al., (1998) Cell 92: 773-784; Halbrook and Scheel, (1989) Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 347-369; Lamb et al., (1997) Ann. Rev. Plant Mol. Biol. Plant Physiol. 48: 251-275; Lapwood et al. (1984) Plant Pathol. 33: 13-20; Levine et al. (1996) Curr. Biol. 6: 427-437; McKersie et al., (2000) Plant Physiol. 122(4): 1427-1437; Olson and Varner (1993) Plant J. 4: 887-892; Pastore et al., (2000), FEBS Lett 470(1): 88-92; Pastori et al., (1997) Plant Physiol. 113: 411-418.Romero-Puertas et al., (1999) Free Radic. Res. 1999 31 Suppl: S25-31; Shirataki et al., Anticancer Res 20(1A): 423-426 (2000); Wu et al., (1995) Plant Cell 7: 1357-1368;

III.E.6.b. Use Of Reactive Oxygen Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the reactive oxygen responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations above and included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Reinforcement of Cell Walls	Modulation Of The Production Of ExtracTable Proline-Rich Protein	Bradley et al. 1992. Cell 70, 21-30
	Modulation Of Lignification	Mansouri et al. (1999) Physiol Plant 106: 355-362
Stress, Disease, Pathogen Resistance and Wounding	Induction Of Pathogenesis Related Proteins, Phytoalexins And Many Defense Pathways.  Induction Of Detoxifying Enzymes Such As Glutathione S-Transferase And Ascorbate Peroxidase  Disease Resistance	Chamnongpol et.al.(1998) Proc. Natl.Acad Sci USA 12;95:5818-23.  Davis et al. (1993) Phytochemistry 32: 607-611.  Chen et.al. Plant J. (1996) 10:955-966  Gadea et.al.(1999) Mol Gen Genet 262:212-219  Wu et.al.(1995) Plant Cell 7: 1357-68

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Reactive Oxygen Generation Following Wounding And Changes In Physical Pressure	Orozco-Cardenas and Ryan (1999) Proc.Nat. Acad. Sci. USA 25;96:6553-7. Yahraus et al. (1995) Plant Physiol. 109: 1259-1266
	Modulation Of Genes Involved In Wound Repair And Cell Division	LEGENDRE ET AL. (1993) PLANT PHYSIOL. 102: 233-240
	Modulation Of Nitric Oxide Signaling	DELLEDONNE ET AL. (1998) NATURE 394: 585-588
	Salicyclic Acid Accumulation And Signaling	DURNER AND KLESSIG (1996) J.BIOL.CHEM. 271:28492-501
Programmed Cell Death	Induction Of Cell Death Pathway Genes	LEVINE ET AL. (1996) CURR. BIOL. 6: 427-437. REYNOLDS ET.AL.(1998) BIOCHEM.J. 330:115-20

Other biological activities that can be modulated by the reactive oxygen responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Reactive oxygen responsive genes are characteristically differentially transcribed in response to fluctuating reactive oxygen levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various reactive oxygen responsive genes in the aerial parts of a plant at 1 and 6 hours after the plant was

sprayed with Silwett L-77 solution enriched with hydrogen peroxide as compared to plants sprayed with Silwett L-77 alone.

The data from this time course reveal a number of types of reactive oxygen responsive genes and gene products, including "early responders," and "delayed responders". Profiles of individual reactive oxygen responsive genes are shown in the Table below together with examples of which associated biological activities are modulated when the activities of one or more such genes vary in plants.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
Upregulated transcripts (Higher at 1h Than 6h) (Level at 1 h ≈ 6h)	Early Responders To Reactive Oxygen	- Perceiving Reactive Oxygen - Reactive Oxygen Response Transduction Pathways  - Initiating Specific Gene Transcription	-Transcription Factors -Kinases And Phosphatases -Transporters - Glutathione S-Transferase -Heat Shock Proteins -Salicylic Acid Response Pathway Proteins -Jasmonic Acid Pathway Proteins -Dehydrins -Peroxidases -Catalase -Proteases -Pathogen Response Proteins -Ca 2+ Channel Blockers -Phenylalanine Ammonia Lyase
Upregulated transcripts (Lower at 1h Than 6h)	Delayed Reactive Oxygen Responders	Maintenance Of Defence Pathways To Control Active Oxygen  Activation Of Cell	-Transcription Factors - Kinases And Phosphatases - Reactive Oxygen Scavenging Enzymes - Cell Wall And Cell Division/Growth Promoting Pathway Enzymes

		Death Pathways In Specific Cells	<ul style="list-style-type: none"> <li>- Pathogen Response Proteins</li> <li>- Proteins Of Defence Pathways</li> <li>- Proteases, Cellulases, Nucleases And Other Degrading Enzymes.</li> <li>- Membrane Proteins</li> <li>-Mitochondrial And Chloroplast Energy Related Proteins</li> </ul>
Downregulated transcripts  Level at 1h $\geq$ 6h  Level at 6h > 1h.	Early Responder  Repressors Of Reactive Oxygen Response Pathways   Genes Of Pathways That Are Minimized In Response To Reactive Oxygen	Negative Regulation Of Reactive Oxygen-Inducible Pathways Released   Reduction In Activities Of Pathways Not Maintained Under High Reactive Oxygen	<ul style="list-style-type: none"> <li>-Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>- Chromatin Remodelling Proteins</li> <li>- Metabolic Enzymes In Affected Cells</li> <li>- Membrane Proteins And Cell Wall Proteins</li> <li>-Transcription Factors</li> <li>- Kinases And Phosphatases</li> <li>-Chromatin Remodelling Proteins</li> <li>- Metabolic Enzymes In Affected Cells</li> <li>- Membrane Proteins And</li> </ul>
Down Regulated Transcripts  (Level at 1h > 6 h)	Delayed Responder  Repressors Of Reactive Oxygen Response Pathways   Genes Of	Negative Regulation Of Reactive Oxygen Inducible Pathways Released	

	Pathways That Are Minimised In Response To Reactive Oxygen	Reduction In Activities Of Pathways Not Maintained Under Reactive Oxygen  Programmed Cell Death	Cell Wall Proteins  - Many Proteins In Cells Undergoing Cell Death Or In Damaged Cells
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Further, promoters of reactive oxygen responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by reactive oxygen or any of the following phenotypes or biological activities below.

### **III.E.7. SALICYLIC ACID RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Plant defense responses can be divided into two groups: constitutive and induced. Salicylic acid (SA) is a signaling molecule necessary for activation of the plant induced defense system known as systemic acquired resistance or SAR. This response, which is triggered by prior exposure to avirulent pathogens, is long lasting and provides protection against a broad spectrum of pathogens. Another induced defense system is the hypersensitive response (HR). HR is far more rapid, occurs at the sites of pathogen (avirulent pathogens) entry and precedes SAR. SA is also the key signaling molecule for this defense pathway.

Changes in SA concentration in the surrounding environment or within a plant results in modulation of many genes and gene products. Examples of such SA responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to SA treatment.

While SA responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different SA responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of SA responsive polynucleotides and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress and pathogen induced pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common and overlapping pathways.

Such SA responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in SA concentration or in the absence of SA fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108586, 108587, 108515, 108552, 108471, 108472, 108469, 108470, 107953, 107960, 108443, 108440, 108441, 108475, 108476). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

SA genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### SA Genes Identified By Cluster Analyses Of Differential Expression

#### SA Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of SA genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108586, 108587, 108515, 108552, 108471, 108472, 108469, 108470, 107953, 107960, 108443, 108440, 108441, 108475, 108476 of the MA\_diff table(s).

SA Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of SA genes. A group in the MA\_clust is considered a SA pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

SA Genes Identified By Amino Acid Sequence Similarity

SA genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis SA genes. Groups of SA genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a SA pathway or network is a group of proteins that also exhibits SA functions/utilities.

Further, promoters of SA responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by SA or any of the following phenotypes or biological activities below.

III.E.7.a. Use Of Salicylic Acid-Responsive Genes To Modulate Phenotypes

SA responsive genes and gene products are useful to or modulate one or more phenotypes including pathogen tolerance and/or resistance; Avr/R locus Interactions; non-host interactions; HR; SAR, e.g., SA responsive genes and/or products in conjunction with any of the organisms listed below; resistance to bacteria e.g. to *Erwinia stewartii*, *Pseudomonas syringae*, *Pseudomonas tabaci*, Stuart's wilt, etc.; resistance to fungi e.g. to Downy mildews such as *Sclerophthora macrospora*, *Sclerophthora rayissiae*, *Sclerospora graminicola*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora sacchari*, *Peronosclerospora maydis*; rusts such as *Puccinia sorphi*, *Puccinia polysora*, *Physopella zeae*, etc.; and to other fungal

diseases e.g. Cercospora zeae-maydis, Colletotrichum graminicola, Fusarium moniliforme, Exserohilum turcicum, Bipolaris maydis, Phytophthora parasitica, Peronospora tabacina, Septoria, etc.; resistance to viruses or viroids e.g., to Tobacco or Cucumber Mosaic Virus, Ringspot Virus, Necrosis Virus, Pelargonium Leaf Curl Virus, Red Clover Mottle Virus, Tomato Bushy Stunt Virus, and like viruses; resistance to insects, such as to aphids e.g. Myzus persicae; to beetles and beetle larvae; to lepidoptera larvae e.g. Heliothis etc.; resistance to nematodes, e.g. Meloidogyne incognita etc.; local resistance in primary (infected) or secondary (uninfected) leaves; stress tolerance; winter survival; cold tolerance; salt tolerance; heavy metal tolerance, such as cadmium; tolerance to physical wounding; increased organelle tolerance to redox stress (such as in mitochondria, and chloroplasts); cell death; programmed cell death, including death of diseased tissue and during senescence); fruit drop; biomass; fresh and dry weight during any time in plant life, such as maturation; number of flowers, seeds, branches, and/or leaves; seed yield, including number, size, weight, and/or harvest index; fruit yield, including number, size, weight, and/or harvest index; plant development; time to fruit maturity; cell wall strengthening and reinforcement; plant product quality; e.g. paper making quality); food additives; treatment of indications modulated by free radicals; and cancer.

To regulate any of the desired phenotype(s) above, activities of one or more of the SA responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Zhao et al. (1998, Plant Cell 10:359-70) and Alvarez et al. (1998, Cell 92: 733-84).

III.E.7.b. Use Of Salicylic Acid-Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the SA responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below.

PATENT  
Attorney Dkt. 2750-1571P  
Filed August 22, 2003

Such biological activities can be measured according to the citations included in the  
Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATION INCLUDING ASSAYS
Protection From Microbial Pathogens	Systemic Acquired Resistance (SAR)  - Phytoalexin Biosynthesis  - PR Protein Biosynthesis  Local Resistance  Wound Response	Alvarez et al. (1998) Cell 92: 733-84  Lapwood et al. (1984) Plant Pathol. 33: 13-20  Davis et al. (1993) Phytochemistry 32: 607-11  Yahraus et al. (1995) Plant Physiol. 109: 1259-66
Cell Signaling	- Modulation Of Reactive Oxygen Signaling  - Modulation Of No Signaling	-Alvarez et al. (1998) Cell 92: 773-784  Delledonne et al. (1998) Nature 394: 585-588
Growth And Development	- Lignification	Redman et al. (1999) Plant Physiol. 119: 795-804

Other biological activities that can be modulated by the SA responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Salicylic acid responsive genes are characteristically differentially transcribed in response to fluctuating SA levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table reports the changes in transcript levels of various SA responsive genes in entire seedlings at 1 and 6 hours after the seedling was sprayed with a Hoagland's solution enriched with SA as compared to seedlings sprayed with Hoagland's solution only.

The data from this time course can be used to identify a number of types of SA responsive genes and gene products, including "early responders" and "delayed responders."

Profiles of these different SA responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Upregulated Genes (Level At 1h $\geq$ 6h) Or (Level At 1h > 6h)	Early Responders To SA	- SA Perception - SA Uptake - Modulation Of SA Response Transduction Pathways	-Transcription Factors -Transporters, Kinases, Phosphatases, G-Proteins, LRR, DNA Remodelling Proteins
Upregulated Genes (Level At 1h < 6h)	Delayed Responders To SA	- Specific Defense gene Transcription Initiation (E.G. Pr Genes, Pal)	-Proteases, PRProteins, Cellulases, Chitinases, Cutinases, Other Degrading Enzymes, Pal, Proteins Of Defense Pathways, Cell Wall Proteins  Epoxide Hydrolases, Methyl Transferases
Downregulated (Level At 1h $\geq$ 6h) Or (Level At 6h > 1h)	- Early Responder Repressors To SA - Genes With Discontinued	- Negative Regulation Of SA Inducible Pathways Released	Transcription factors, kinases, phosphatases, G-proteins, LRR, transporters, calcium binding proteins, chromatin remodelling

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
	Expression Or Unstable mRNA In The Presence Of SA		protein
Down-Regulated Transcripts (Level At 1h > 6h)	- Delayed Responders To SA Metabolism - Genes With Discontinued Expression Or Unstable mRNA In The Presence Of SA	Negative Regulation Of SA Inducible Pathways Released	Transcription Factors, Kinases, Phosphatases, G-Proteins, LRR, Transporters, Calcium Binding Proteins, Chromatin Remodelling Protein

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the SA responsive genes when the desired sequence is operably linked to a promoter of a SA responsive gene.

### III.E.8. NITRIC OXIDE RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

The rate-limiting element in plant growth and yield is often its ability to tolerate suboptimal or stress conditions, including pathogen attack conditions, wounding and the presence of various other factors. To combat such conditions, plant cells deploy a battery of inducible defense responses, including synergistic interactions between nitric oxide (NO), reactive oxygen intermediates (ROS), and salicylic acid (SA). NO has been shown to play a critical role in the activation of innate immune and inflammatory responses in animals. At least

part of this mammalian signaling pathway is present in plants, where NO is known to potentiate the hypersensitive response (HR). In addition, NO is a stimulator molecule in plant photomorphogenesis.

Changes in nitric oxide concentration in the internal or surrounding environment, or in contact with a plant, results in modulation of many genes and gene products. Examples of such nitric oxide responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. They were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to nitric oxide treatment.

While nitric oxide responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different nitric oxide responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of the levels of such proteins is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a nitric oxide responsive polynucleotide and/or gene product with other environmentally responsive polynucleotides is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, pathogen stimulated pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108584, 108585, 108526, 108527, 108559). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

NO genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

PATENT  
Attorney Dkt. 2750-1571P  
Filed August 22, 2003

NO Genes Identified By Cluster Analyses Of Differential Expression

NO Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of NO genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108584, 108585, 108526, 108527, 108559 of the MA\_diff table(s).

NO Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of NO genes. A group in the MA\_clust is considered a NO pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

NO Genes Identified By Amino Acid Sequence Similarity

NO genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis NO genes. Groups of NO genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a NO pathway or network is a group of proteins that also exhibits NO functions/utilities.

Such nitric oxide responsive genes and gene products can function either to increase or dampen the above phenotypes or activities either in response to changes in nitric oxide concentration or in the absence of nitric oxide fluctuations. Further, promoters of nitric oxide responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by nitric oxide or any of the following phenotypes or biological activities below.

III.E.8.a. Use Of Nitric Oxide-Responsive Genes To Modulate Phenotypes :

Nitric oxide responsive genes and gene products are useful to or modulate one or more phenotypes including Stress Responses, Mediation of response to stresses, Disease resistance, Growth, Roots, Stems, Leaves, Cells, Promotes leaf cell elongation, Biomass; Fresh and Dry Weight during any time in plant life, such as at maturation; Size and/or Weight; Flowers, Seeds, Branches, Leaves, Roots, Development, Seed Development, Dormancy; Control rate and timing of germination, Prolongs seed storage and viability; and Senescence.

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the nitric responsive genes when the desired sequence is operably linked to a promoter of a nitric responsive gene.

To regulate any of the desired phenotype(s) above, activities of one or more of the nitric oxide responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998) Methods. Mol. Biol. 82: 259-266 and/or screened for variants as described in Winkler et al. (1998) Plant Physiol. 118: 743-50 and visually inspected for the desired phenotype. Alternatively, plants can be metabolically and/or functionally assayed according to Beligni and Lamattina (2000) Planta 210: 215-21), Lapwood et al (1984) Plant Pathol 33: 13-20, and/or Brown and Botstein (1999) Nature Genet. 21: 33-37.

III.E.8.b. Use Of Nitric Oxide-Responsive Genes To Modulate Biochemical Activities:

The activities of one or more of the nitric oxide responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Stress Response	-Programmed Cell Death  -Reactive Oxygen based Defence Pathways	Levine et al (1996) Curr. Biol 6: 427-37  Sellins and Cohen (1991) Radiat. Res. 126: 88-95  Kumar and Klessig (2000) Mol. Plant Microbe Interact. 13:347-351

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Disease Resistance	-Microbial Pathogen resistance pathways  -Programmed Cell Death  -Cellular Protectant Gene expression  - Phytoalexin Biosynthesis	Lapwood et al (1984) Plant Pathol 33: 13-20  Kumar and Klessig (2000) Mol. Plant microbe interact.13: 347-351  Klessig et.al.(2000) Proc. Nat. Acad. Sci USA 97: 8849-8855  Delledonna et al(1998) Nature 394: 585-588   Levine et al (1996) Curr. Biol 6: 427-437  Sellins and Cohen (1991) Radiat. Res. 126: 88-95   Brown and Botstein (1999) Nat Genet 21: 33-37   Davis et al. (1993) Phytochemistry 32: 607-611

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Signal Transduction	Regulation of hydrogen peroxide signaling	Wu et al. (1995) Plant Cell 7, 1357-1368
Reorientation of nitrogen metabolism	Induction of ribosomal proteins, asparagine synthesis, proteases, RNases	This study. Standard assays for detection of changes
Reorientation of sugar and energy metabolism	Induction of sugar transporters, ATPases, glycohydrolases, and glycolytic enzymes, for example	This study. Standard assays for detection of changes

Other biological activities that can be modulated by the NO responsive genes and gene products are listed in the Reference Tables. Assays for detecting such biological activities are described in the Protein Domain table.

NO responsive genes are characteristically differentially transcribed in response to fluctuating NO levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various NO responsive genes in aerial tissues at 1 and 6 hours after a plant was sprayed with a Silwett L-77 solution enriched with 5 mM sodium nitroprusside, which is an NO donor. These changes are in comparison with plants sprayed with Silwett L-77 solution only.

The data from this time course can be used to identify a number of types of NO responsive genes and gene products, including "early responders" and "delayed responders". Profiles of these different nitric oxide responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVEL	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Upregulated genes (level at 1 hour $\geq$ 6 hours)  (level at 1 hour > 6 hours)	Early responder repressors to NO	<ul style="list-style-type: none"> <li>- NO Perception</li> <li>- NO Uptake</li> <li>- Modulation of NO Response Transduction Pathways</li> <li>Specific Gene Transcription Initiation of Pathways to Optimize NO Response Pathways</li> </ul>	<ul style="list-style-type: none"> <li>-Transcription Factors</li> <li>-Transporters</li> <li>-Pathogen responsive proteins, salicylic and jasmonate pathway proteins</li> <li>-Proteins to provide defence against active oxygen e.g. glutathione transferase, ascorbate free radical reductase, ascorbate peroxidase, nitrilase, heat shock proteins</li> <li>-Proteins to reorient metabolism e.g. proteases, RNases, proteasomes, asparagine synthetase, glycohydrolases, transporters</li> <li>-Proteins to inhibit transport of nitric oxide</li> <li>-Degradation enzymes</li> </ul>

GENE EXPRESSION LEVEL	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Upregulated transcripts (level at 1 hour < 6 hours)	Delayed NO responders	<ul style="list-style-type: none"> <li>- Maintenance of metabolism in presence of High NO</li> <li>- Maintenance of disease defence pathways</li> <li>- Maintenance of pathways against reactive oxygen production</li> <li>Maintenance of different metabolic programs</li> <li>Selective cell death</li> </ul>	<ul style="list-style-type: none"> <li>- NO Metabolic Pathway enzymes</li> <li>- Pathogen responsive proteins, salicylic and jasmonate pathway proteins</li> <li>- Proteins to provide defence against active oxygen e.g. glutathione transferase, ascorbate free radical reductase, ascorbate peroxidase, nitrilase, heat shock proteins</li> <li>- Proteins to reorient and sustain metabolism e.g. proteases, RNases, proteasomes, asparagine synthetase, glycohydrolases, transporters,</li> <li>- Proteins to inhibit transport of NO</li> <li>- Degradation enzymes</li> </ul>
Down Regulated	-Early responders of	Negative regulation of	-Transcription factors

GENE EXPRESSION LEVEL	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Transcripts (level at 1 hours $\geq$ 6 hours) (level at 6 hours > 1 hour)	NO utilization pathways  -Genes with discontinued expression or unsTable mRNA following nitric oxide uptake	NO utilization pathways released  Reorientation of metabolism  Programmed-cell death	-Kinases and phosphatases -Chromatin restructuring proteins - Transcription factors, metabolic enzymes, kinases and phosphatases, transporters, ribosomal proteins  -Most proteins in cells undergoing cell death
Down Regulated Transcripts (level at 1 hour > 6 hours)	-Delayed responder repressors of NO stress metabolism  -Genes with discontinued expression or unsTable mRNA following nitric oxide uptake	Negative regulation of NO utilization pathways released  Reorientation of metabolism	Transcription factors -Kinases and phosphatases -Chromatin restructuring proteins  -Transcription factors, metabolic enzymes, kinases and phosphatases, transporters, ribosomal proteins.

GENE EXPRESSION LEVEL	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
		Programmed cell death	-Most proteins in cells undergoing programmed cell death

#### USE OF PROMOTERS OF NO RESPONSIVE GENES

Promoters of NO responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the NO responsive genes where the desired sequence is operably linked to a promoter of a NO responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.9. OSMOTIC STRESS RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

The ability to endure and recover from osmotic and salt related stress is a major determinant of the geographical distribution and productivity of agricultural crops. Osmotic stress is a major component of stress imposed by saline soil and water deficit. Decreases in yield and crop failure frequently occur as a result of aberrant or transient environmental stress conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the osmotic and salt tolerance of a crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased osmotic tolerance would provide a more reliable means to minimize crop losses and diminish the use of energy-costly practices to modify the soil environment.

Changes in the osmotic concentration of the surrounding environment or within a plant results in modulation of many genes and gene products. Examples of such osmotic stress

responsive genes and gene products, including salt responsive genes, are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, MA\_diff and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While osmotic and/or salt stress responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different osmotic stress responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of an osmotic stress responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in a common pathway.

Such osmotic and/or salt stress responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in osmotic concentration or in the absence of osmotic fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: 108570, 108571, 108541, 108542, 108553, 108539, 108540). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Osmotic Stress genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Osmotic Stress Genes Identified By Cluster Analyses Of Differential Expression

Osmotic Stress Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Osmotic Stress genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID 108570, 108571, 108541, 108542, 108553, 108539, 108540 of the MA\_diff table(s).

Osmotic Stress Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Osmotic Stress genes. A group in the MA\_clust is considered a Osmotic Stress pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Osmotic Stress Genes Identified By Amino Acid Sequence Similarity

Osmotic Stress genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Osmotic Stress genes. Groups of Osmotic Stress genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Osmotic Stress pathway or network is a group of proteins that also exhibits Osmotic Stress functions/utilities.

Further, promoters of osmotic stress responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by osmotic stress or any of the following phenotypes or biological activities below.

III.E.9.a. Use Of Osmotic Stress Responsive Genes To Modulate Phenotypes

Osmotic stress responsive genes and gene products are useful to or modulate one or more phenotypes including growth; roots; stems; leaves; development (such as cell growth by DNA synthesis and cell division, seed development (with regard to desiccation tolerance and dormancy, such as control rate of germination and prolongs seed storage and viability and senescence); stress responses; desiccation; drought; and salt.

To regulate any of the phenotype(s) above, activities of one or more of the osmotic stress responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to de Castro (1998, Phytochemistry 47: 689-694), Xu (1998, J Exp Bot 49: 573-582), Ausubel et al. (In: Current Protocols in Molecular Biology (1999) Volume 1, chapter 4, eds. Ausubel, Brent, Kingston, Moore, Seidman, Smith and Struhl, New York, NY) and De Castro et al. (2000, Plant Physiol 122: 327-36)

III.E.9.b. Use Of Osmotic Stress Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the osmotic stress responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cell Growth And Differentiation	Regulation Of Osmolyte Synthesis	Yoshii et al. (1995) The Plant Journal

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
		7: 751-60
	Regulation Of Glycolate Pathway And Photoinhibition Of Photosystem II In Response To Stress	Streb et al. (1993) Physiologia Plantarum. 88:590-598
Gene Regulation	Transcriptional Regulation Of Osmotic Stress Induced Proteins Through DNA Binding Proteins	Current Protocols in Molecular Biology / edited by Frederick M. Ausubel .. [et al.]. New York : Published by Greene Pub. Associates and Wiley-Interscience : J. Wiley, c1987
	Transcriptional Regulation Of Osmotic Stress Induced Proteins Through Protein Phosphorylation And Dephosphorylation	Jonak (1996) Proceedings of the National Academy of Sciences of the United States of America, 93: 11274-11279; Monroy, A. et al., (1998) Analytical Biochemistry 265: 183-185;
	Regulation Of Osmotic Stress Induced Gene Protein Accumulation By Protein Protein Interaction Between Osmotic Stress Regulated Genes And	McCright (1998) IN: Methods in Molecular Biology; Protein phosphatase protocols; Ludlow (1998) Humana

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Protein Phosphatase 2C	Press Inc. ; Suite 808, 999 Riverview Drive, Totowa, New Jersey 07512, USA. :263-277.
	Transcriptional Regulation Of Heat Induced Genes Through Chromatin Remodeling	Luo and Dean (1999) Journal of the National Cancer Institute 91: 1288-1294; Chromatin protocols (1999) edited by Peter B. Becker. Totowa, N.J. : Humana Press
	Activity Of Abcisic Acid Regulated DNA Binding Proteins	Gubler et al. (1999) Plant Journal 17: 1-9
	Accumulation Of RNA Binding Proteins That Regulate Osmotic Stress	Sato (1995) Nucleic Acids Research 23: 2161-2167.
Stress Response	Synthesis And Metabolism Of Osmoprotectants Such As Betaine, Proline And Trehalase	Minocha et al. (1999) Plant Physiol and Biochem 37: 597-603
	Regulation Of Sugar Transporters	Dejardin et al.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
		(1999) Biochem J; 344 Pt 2:503-9
	Regulation Of Vacuolar Sodium/Proton Antiport Activity And The Detoxification Of Cations	Gaxiola et al. (1999) PNAS USA 96: 1480-1485
	Regulation Of Intracellular Na+ And Li+ Ion Concentrations	Espinosa-Ruiz et al. (1999) The Plant Journal 20: 529-539
	Regulation Of Universal Stress Protein Homologue Activity By Phosphorylation And Dephosphorylation.	Freestone et al. (1997) Journal of Molecular Biology, v. 274: 318-324
	Regulation/Maintenance Of Protein Stability During Thermal Stress	Walker (1996) Humana Press Inc. Suite 808, 999 Riverview Drive, Totowa, New Jersey 07512, USA
	Regulation Of Protein Degradation During Thermal Stress.	Vierstra (1996) Plant Molecular Biology,32:275-302.  Vierstra and Callis (1999) Plant Molecular Biology, 41:435-442

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Signal Transduction	Activation Of Stress Response Genes	Xinong et al. (1999) The Plant Journal 19: 569-578
	Salt Tolerance	Piao (1999) Plant Physiol 19: 1527-1534
	Calcium Mediated Stress Response	Subbaiah et al. (1994) Plant Physiology 105:369-376 Kudla et al. (1999) PNAS USA 96: 4718-4723

Other biological activities that can be modulated by the osmotic stress responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Osmotic stress responsive genes are characteristically differentially transcribed in response to fluctuating osmotic stress levels or concentrations, whether internal or external to an organism or cell. MA\_diff table reports the changes in transcript levels of various osmotic stress responsive genes in aerial tissues of plants at 1 and 6 hours after the plants were sprayed with Hoagland's solution containing 20% PEG as compared to aerial tissues from plants sprayed with Hoagland's solution only.

The data from this time course can be used to identify a number of types of osmotic stress responsive genes and gene products, including "early responding," "sustained osmotic stress responders," "repressors of osmotic stress pathways" and "osmotic stress responders." Profiles

of these different osmotic stress responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITIES OF GENE PRODUCTS
Up Regulated Transcripts (Level At 1 Hour $\geq$ 6 Hours) (Level At 1 Hour > 6 Hours)	<ul style="list-style-type: none"> <li>• Early Responders To Osmotic Stress</li> <li>• Universal Stress Response Genes</li> <li>• Osmotic Stress Responders</li> <li>• Abscisic Acid Biosynthesis And Perception</li> </ul>	<ul style="list-style-type: none"> <li>• Osmotic Stress Perception</li> <li>• Osmolyte Uptake</li> <li>• Modulation Of Osmotic Stress Response Signal Transduction Pathways</li> <li>• Specific Gene Transcription Initiation</li> <li>• Specific Gene Transcription Repression</li> <li>• Translation Activation</li> <li>• Translation Repression</li> <li>• Repression Of “Normal State” Pathways To Optimize Osmotic Stress Response</li> <li>• Activation Of Stress Signaling Pathways</li> <li>• Up Regulation Of</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription Factors</li> <li>• Transcription Coactivators</li> <li>• Membrane Transporters</li> <li>• Proline Biosynthesis</li> <li>• Selective Inhibition Of Osmolyte Transport</li> <li>• Protein Ubiquitination</li> <li>• Protein Degradation</li> <li>• Rna Binding Proteins</li> <li>• Modification Of Protein Activity By Phosphatases, Kinases</li> <li>• Synthesis And Or Activation Of Oxide Hydrolases,</li> </ul>

		<p>Abscisic Acid Biosynthesis Pathway Protein Accumulation And Activity</p> <ul style="list-style-type: none"><li>• Scavenging Reactive Oxygen Species</li><li>• Modification Of Cell Wall Composition</li><li>• Up-Regulation Of Universal Stress Response Protein Accumulation</li></ul>	<p>Suoeroxidedismutase, Iron Ascorbate Peroxidase</p> <ul style="list-style-type: none"><li>• Activation Of Signaling Pathway By Calcium Binding Proteins,</li><li>• Modification Of Protein Activity By Protein-Protein Interaction</li><li>• Change In Chromatin Structure And/Or Localized Dna Topology</li><li>• Modification Of Pre-Existing Translation Factors By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li><li>• Synthesis Of New Translation Factors</li><li>• Abscisic Acid Biosynthesis</li></ul>
Up Regulated Transcripts (Level At 1 Hr < 6	<ul style="list-style-type: none"><li>• Sustained Osmotic Stress Responders</li></ul>	<ul style="list-style-type: none"><li>• Osmolyte Adjustment And Adaptation</li><li>• Photosynthetic</li></ul>	<ul style="list-style-type: none"><li>• Osmotic Stress Metabolic Pathways</li></ul>

Hr)	<ul style="list-style-type: none"> <li>• Repressor Of Osmotic Stress Pathways</li> <li>• Abscisic Acid Perception, Biosynthesis And Regulation</li> </ul>	<ul style="list-style-type: none"> <li>Activity Modification</li> <li>• Activation Of “Normal State” Biosynthesis Genes</li> <li>• Negative Regulation Of Osmotic Stress Pathways</li> <li>• Negative Regulation Of Abscisic Acid Biosynthesis</li> <li>• Activation Of Abscisic Acid Degradation Pathway</li> <li>• Cell Wall Composition Modification</li> </ul>	<ul style="list-style-type: none"> <li>• Sugar Biosynthetic Pathways</li> <li>• Sugar Transporters</li> <li>• Transcription Factors</li> <li>• Transcription Coactivators</li> <li>• Membrane Transporters</li> <li>• Abscisic Acid Biosynthesis</li> </ul>
Down-Regulated Transcripts (Level At 1 Hr ≈ 6 Hr) (Level At 6 Hr > 1 Hr)	<p>Early Responder Repressors Of "Normal" State Of Metabolism</p> <p>Negative Regulators Of Abscisic Acid Biosynthesis And Perception.</p> <p>Positive Regulators Of “Normal State” Metabolic Pathways.</p>	<ul style="list-style-type: none"> <li>Metabolic Repression</li> <li>Specific Gene Transcription Initiation</li> <li>Specific Gene Transcription Repression</li> <li>Translation Activation</li> <li>Translation Repression</li> <li>Abscisic Acid Degradation</li> <li>Protein Degradation</li> </ul>	<ul style="list-style-type: none"> <li>Transcription Factors</li> <li>Transcription Coactivators</li> <li>Protein Degradation</li> <li>Rna Binding Proteins</li> <li>Modification Of Protein Activity By Phosphatases, Kinases</li> <li>Activation Of Signaling Pathway</li> </ul>

			<p>By Calcium Binding Proteins,</p> <ul style="list-style-type: none"><li>• Modification Of Protein Activity By Protein-Protein Interaction</li><li>• Change In Chromatin Structure And/Or Localized Dna Topology</li><li>• Modification Of Pre-Existing Translation Factors By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li><li>• Synthesis Of New Translation Factors</li></ul>
Down-Regulated Transcripts (Level At 1 Hr > 6 Hr)	Repressors Of "Normal" State Of Metabolism  Genes With Discontinued Expression Or Unstable mRNA In Presence Of Osmotic Stress	<ul style="list-style-type: none"><li>• Osmotic Stress Adaptation</li><li>• Negative Regulation Of Abscisic Acid Biosynthesis</li><li>• Negative Regulation Of Osmotic Stress Response Pathways</li><li>• Genes</li><li>• Osmolyte Synthesis</li></ul>	<ul style="list-style-type: none"><li>• Transcription Factors</li><li>• Transcription Coactivators</li><li>• Protein Degradation</li><li>• Rna Binding Proteins</li><li>• Modification Of Protein Activity By</li></ul>

Repressor Of Osmotic Stress Pathways  Repressors Of Abscisic Acid Biosynthesis, Perception And Regulation	And Osmolyte Cellular Partitioning Readjustment Activation Of “Normal State” Metabolic Pathways		Phosphatases, Kinases <ul style="list-style-type: none"><li>• Activation Of Signaling Pathway By Calcium Binding Proteins,</li><li>• Modification Of Protein Activity By Protein-Protein Interaction</li><li>• Change In Chromatin Structure And/Or Localized Dna Topology</li><li>• Modification Of Pre-Existing Translation Factors By Phosphorylation (Kinases) Or Dephosphorylation (Phosphatases)</li><li>• Synthesis Of New Translation Factors</li><li>• Sugar Biosynthetic Pathways</li><li>• Sugar Transporters</li></ul>
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Further, any desired sequence can be transcribed in similar temporal, tissue, or

environmentally specific patterns as the osmotic stress responsive genes when the desired sequence is operably linked to a promoter of an osmotic stress responsive gene.

### III.E.10. ALUMINUM RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Aluminum is toxic to plants in soluble form ( $\text{Al}^{3+}$ ). Plants grown under aluminum stress have inhibited root growth and function due to reduced cell elongation, inhibited cell division and metabolic interference. As an example, protein inactivation frequently results from displacement of the  $\text{Mg}^{2+}$  cofactor with aluminum. These types of consequences result in poor nutrient and water uptake. In addition, because stress perception and response occur in the root apex, aluminum exposure leads to the release of organic acids, such as citrate, from the root as the plant attempts to prevent aluminum uptake.

The ability to endure soluble aluminum is a major determinant of the geographical distribution and productivity of agricultural crops. Decreases in yield and crop failure frequently occur as a result of aberrant, hot conditions even in areas considered suitable for the cultivation of a given species or cultivar. Only modest increases in the aluminum tolerance of crop species would have a dramatic impact on agricultural productivity. The development of genotypes with increased aluminum tolerance would provide a more reliable means to minimize crop losses and diminish the use of costly practices to modify the environment.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) *Science* **270**: 467-70). The Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing 10,000 non-redundant ESTs, selected from 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full-length cDNA and genomic sequence databanks, and identical Ceres clones identified. MA\_diff table reports the results of this

analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which are aluminum response responsive genes.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Aluminum (relating to SMD 7304, SMD 7305)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Aluminum genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Aluminum Genes Identified By Cluster Analyses Of Differential Expression

Aluminum Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Aluminum genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Aluminum (relating to SMD 7304, SMD 7305) of the MA\_diff table(s).

Aluminum Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Aluminum genes. A group in the MA\_clust is considered a Aluminum pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Aluminum Genes Identified By Amino Acid Sequence Similarity

Aluminum genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Aluminum genes. Groups of Aluminum genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Aluminum pathway or network is a group of proteins that also exhibits Aluminum functions/utilities.

III.E.10.a. Use Of Aluminum Response Genes To Modulate Phenotypes

Changes in aluminum concentrations in a plant's surrounding environment results in modulation of many genes and gene products. Examples of such aluminum response genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While aluminum responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different aluminum responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of a aluminum responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in a common pathway.

Such aluminum responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either

- in response to changes in aluminum concentration or
- in the absence of aluminum fluctuations.

More specifically, aluminum responsive genes and gene products are useful to or modulate one or more phenotypes including growth; roots (such as inhibition of root elongation); stems; leaves; whole plant; development (such as cell growth, elongation, and division) and

mediates response to oxidative stress, calcium-mediated defense, antioxidant defense and pathogenesis.

To produce the desired phenotype(s) above, one or more of the aluminum response genes or gene products can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Li and Fleming (1999, FEBS Lett 461: 1-5), Delhaize et al. (1999, J Biol Chem 274: 7082-8), Sigimoto and Sakamoto (1997, Genes Genet Syst 72: 311-6), Esaki et al. (2000, Plant Physiol 122: 657-65), Leonard and Gerber (1988, Mutat Res 196: 247-57), Baisakhi et al. (2000, Mutat Res 465: 1-9), Ma (2000, Plant Cell Physiol 41: 383-90) and Koyama et al. (1999, Plant Cell 40: 482-8)

Alternatively, the activities of one or more of the aluminum responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

GENERAL CATEGORY	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	ASSAY
Cell Growth and Development	-Phospholipase D (PLD) activity  -Regulation of Phosphatidylserine Synthase (PSS)  -Cell wall strengthening	Toda et al. (1999) Biosci Biotechnol Biochem <u>63</u> : 210-212

		Hamel et al. (1998) <i>Planta</i> 205: 531-38
Stress Response	-Regulation of oxidative stress	Esaki et al. (2000) <i>Plant Physiol</i> 122: 657-655
	-Regulation of antioxidant defense and DNA repair	Baisakhi et al. (2000) <i>Mutat Res</i> 465: 1-9
	-Secretion of Organic Acids (e.g. maleate, citrate) from root apex	Koyama et al. (1999) <i>Plant Cell</i> 40: 482-8
	-Ca <sup>2+</sup> -mediated Defense Responses Against Low pH	Plieth et al. (1999) <i>Plant J</i> 18: 634-50
Signaling	-H <sup>+</sup> transport	Degenhardt et al. (1988) <i>Plant Physiol</i> 117: 19-27
	-Auxin transport	Rashotte et al. (2000) <i>Plant Physiol</i> 122: 481-90

Other biological activities that can be modulated by aluminum response genes and their products are listed in the REFERENCE Table. Assays for detecting such biological activities are described in the Protein Domain table.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	responders to aluminum application	<ul style="list-style-type: none"> <li>• Aluminum perception</li> <li>• Aluminum uptake and transport</li> <li>• Aluminum metabolism</li> <li>• Synthesis of secondary metabolites and/or proteins</li> <li>• Modulation of aluminum response transduction pathways</li> <li>• Specific gene transcription initiation</li> </ul>	<ul style="list-style-type: none"> <li>• Transporters</li> <li>• Metabolic enzymes</li> <li>• Change in cell membrane structure and potential</li> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> <li>• </li> </ul>
Down-regulated transcripts	responder to aluminum repressors of aluminum state of metabolism	<ul style="list-style-type: none"> <li>• Negative regulation of aluminum pathways</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	Genes with discontinued expression or unsTable mRNA in presence of aluminum	<ul style="list-style-type: none"> <li>• Changes in pathways and processes operating in cells</li> <li>• Changes in other metabolisms than aluminum</li> </ul>	<ul style="list-style-type: none"> <li>dephosphorylation (phosphatases)</li> <li>• Change in chromatin structure and/or DNA topology</li> <li>• Stability of factors for protein synthesis and degradation</li> <li>• Metabolic enzymes</li> </ul>

#### USE OF PROMOTERS OF ALUMINUM RESPONSIVE GENES

Promoters of Aluminum responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Aluminum responsive genes where the desired sequence is operably linked to a promoter of a Aluminum responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.E.11. CADMIUM RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Cadmium (Cd) has both toxic and non-toxic effects on plants. Plants exposed to non-toxic concentrations of cadmium are blocked for viral disease due to the inhibition of systemic movement of the virus. Surprisingly, higher, toxic levels of Cd do not inhibit viral systemic

movement, suggesting that cellular factors that interfere with the viral movement are triggered by non-toxic Cd concentrations but repressed in high Cd concentrations. Furthermore, exposure to non-toxic Cd levels appears to reverse posttranslational gene silencing, an inherent plant defense mechanism. Consequently, exploring the effects of Cd exposure has potential for advances in plant disease control in addition to soil bio-remediation and the improvement of plant performance in agriculture.

Changes in cadmium concentrations in a plant's surrounding environment results in modulation of many genes and gene products. Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in plants treated with 10 µM cadmium compared with untreated plants were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and the equivalent Ceres clones identified. The MA\_diff table(s) report(s) the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent cadmium responsive genes.

Examples of such cadmium responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While cadmium responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different cadmium responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore,

manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants. In addition, the combination of a cadmium responsive polynucleotide and/or gene product with other environmentally responsive polynucleotides is also useful because of the interactions that exist between, for example, stress and pathogen induced pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Cadmium (relating to SMD 7427, SMD 7428)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Cadmium genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Cadmium Genes Identified By Cluster Analyses Of Differential Expression

#### Cadmium Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Cadmium genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Cadmium (relating to SMD 7427, SMD 7428) of the MA\_diff table(s).

#### Cadmium Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Cadmium genes. A group in the MA\_clust is considered a Cadmium pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Cadmium Genes Identified By Amino Acid Sequence Similarity

Cadmium genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Cadmium genes. Groups of Cadmium genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Cadmium pathway or network is a group of proteins that also exhibits Cadmium functions/utilities.

Such cadmium responsive genes and gene products can function to either increase or dampen phenotypes or activities either in response to changes in cadmium concentration or in the absence of cadmium fluctuations. Further, promoters of cadmium responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by cadmium or any of the following phenotypes or biological activities below.

III.E.11.a. Use Of Cadmium Responsive Genes, Gene Components  
And Products To Modulate Phenotypes

Cadmium responsive genes and gene products are useful to or modulate one or more phenotypes including growth, roots, initiation and maintenance of cell division, stems, leaves, development, mitochondria, post-embryonic root meristem development, senescence, stress response, modulation of jasmonic acid and other stress control pathways, metabolic detoxification, heavy metals, plant and seed yield; and fruit yield.

Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the cadmium responsive genes when the desired sequence is operably linked to a promoter of a cadmium responsive gene.

To regulate any of the phenotype(s) above, activities of one or more of the cadmium responsive genes or gene products can be modulated and tested by screening for the desired trait.

Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998) Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Ghoshroy et al. (1998, Plant J 13: 591-602), Citovsky et al. (1998, Plant J 16: 13-20), Clemens et al. (1999, EMBO J 18: 3325-33), Chen et al. (2000, Chemosphere 41: 229-34), Xian and Oliver (1998, Plant Cell 10: 1539-90), Romero-Peurtas et al. (1999, Free Rad Res 31: S25-31), Gaur and Noraho (1995, Biomed Environ Sci 8: 202-10), Thomine et al. (2000, PNAS USA 97: 4991-6), Howden et al. (1995, Plant Physiol 107: 1067-73), Kesseler and Brand (1994, Eur J Biochem 225: 907-22) and Vernoux et al. (2000, Plant Cell 12: 97-110).

III.E.10.b. Use Of Cadmium-Responsive Genes, Gene Components  
And Products To Modulate Biochemical Activities

The activities of one or more of the cadmium responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth, Differentiation and Development	Root Growth Initiation and maintenance of cell division Resistance to Cadmium-inhibition of root growth	Thomine et al. (2000) PNAS USA <u>97</u> : 4991-6 Vernoux et al. (2000) Plant Cell <u>12</u> : 97-110
Metabolism	Cadmium sensing	Howden et al. (1995) Plant Physiol <u>107</u> : 1067-73

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Cadmium uptake and transport  Decreased cadmium transport  Phytoremediation	Gaur and Noraho (1995) Biomed Environ Sci <u>8</u> : 202-10  Thomine et al. (2000) PNAS USA <u>97</u> : 4991-6
	Inhibition of oxidative phosphorylation	Kesseler and Brand (1994) Eur. Biochem <u>225</u> : 907-22
Plant Defenses	Viral resistance  Inhibition of systemic movement of virus  Block of viral disease	Ghoshroy et al. (1998) Plant J <u>13</u> : 591-602
	Detoxification of heavy metals	Clemens et al. (1999) EMBO J <u>18</u> : 3325-33
	Enhanced stress resistance	Romero-Peurtas et al. (1999) Free Rad Res <u>31</u> : S25-31
	Cadmium resistance via modulation of jasmonic acid signaling pathway	Xiang and Oliver (1998) Plant Cell <u>10</u> : 1539-90
Signaling	Relief of post-translational gene silencing	Citovsky et al. (1998) Plant J <u>16</u> : 13-20

Other biological activities that can be modulated by the cadmium responsive genes and gene products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Cadmium responsive genes are characteristically differentially transcribed in response to fluctuating cadmium levels or concentrations, whether internal or external to an organism or cell. The MA\_diff table(s) report(s) the changes in transcript levels of various cadmium responsive genes following treatment with 10 µM cadmium, relative to untreated plants. Profiles of some cadmium responsive genes are shown in the Table below together with examples of the kinds of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	Responders to cadmium Application Genes induced by cadmium	Cadmium perception Cadmium uptake and transport Cadmium metabolism Synthesis of secondary metabolites and/or proteins Modulation of cadmium response transduction pathways Specific gene transcription initiation Genes involved in inhibiting systemic movement of plant viral RNA Genes involved in post translational gene silencing	Transporters Metabolic enzymes Change in cell membrane structure and potential Kinases and Phosphatases Transcription activators Change in chromatin structure and/or localized DNA topology RNA binding proteins
Down-regulated	Responders to	Negative regulation of	Transcription factors

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
transcripts	cadmium  Genes repressed by cadmium  Genes with discontinued expression or unstable mRNA in presence of cadmium	cadmium pathways released  Changes in pathways and processes operating in cells  Changes in metabolism other than cadmium pathways  Genes involved in facilitating systemic movement of plant viral RNA  Genes involved in promoting post translational gene silencing	Change in protein structure by phosphorylation (kinases) or Dephosphorylation (phosphatases) Change in chromatin structure and/or DNA topology Factors for protein synthesis and degradation Metabolic enzymes RNA binding proteins

#### USE OF PROMOTERS OF CADMIUM RESPONSIVE GENES

Promoters of Cadmium responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Cadmium responsive genes where the desired sequence is operably linked to a promoter of a Cadmium responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.12. DISEASE RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Often growth and yield are limited by the ability of a plant to tolerate stress conditions, including pathogen attack. To combat such conditions, plant cells deploy a battery of inducible defense responses, including the triggering of an oxidative burst and the transcription of pathogenesis-related protein (PR protein) genes. These responses depend on the recognition of a microbial avirulence gene product (avr) by a plant resistance gene product (R), and a series of downstream signaling events leading to transcription-independent and transcription-dependent disease resistance responses. Reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> and NO from the oxidative burst plays a signaling role, including initiation of the hypersensitive response (HR) and induction of systemic acquired resistance (SAR) to secondary infection by unrelated pathogens. PR proteins are able to degrade the cell walls of invading microorganisms, and phytoalexins are directly microbicidal.

The presence of an avirulent pathogen and/or changes in the concentrations of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and NO in the environment surrounding a plant cell modulate the activities of many genes and, therefore, the levels of many gene products. Examples of tobacco mosaic virus (TMV) responsive genes and gene products, many of them operating through an ROS signaling system, are shown in The Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield. The genes were discovered and characterized from a much larger set by experiments designed to find genes whose mRNA products changed in response to application of TMV to plants.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in response to TMV infection over the non infected controls were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. The MA\_diff table(s) report(s) the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent disease responsive genes.

Manipulation of one or more disease responsive gene activities is useful to modulate the biological processes and/or phenotypes listed below. Disease responsive genes and gene products can act alone or in combination. Useful combinations include disease responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants.

Such disease responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in active oxygen concentration or in the absence of active oxygen fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Disease (relating to SMD 7342, SMD 7343)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Disease genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Disease Genes Identified By Cluster Analyses Of Differential Expression

#### Disease Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The

MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Disease genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Disease (relating to SMD 7342, SMD 7343) of the MA\_diff table(s).

Disease Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Disease genes. A group in the MA\_clust is considered a Disease pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Disease Genes Identified By Amino Acid Sequence Similarity

Disease genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Disease genes. Groups of Disease genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Disease pathway or network is a group of proteins that also exhibits Disease functions/utilities.

Further, promoters of disease responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by disease or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the disease responsive genes when the desired sequence is operably linked to a promoter of a disease responsive gene.

III.E.12.a. Use Of Disease Responsive Genes, Gene Components And Products To Modulate Phenotypes

Disease responsive genes and gene products are useful to or modulate one or more phenotypes including pathogen tolerance and/or resistance; Avr/R locus interactions; non-host interactions;

HR; SAR; resistance to bacteria e.g. to *Erwinia stewartii*, *Pseudomonas syringae*, *Pseudomonas tabaci*, Stuart's wilt, etc.; resistance to fungi , e.g. to downy mildews such as *Scleropthora macrospora*, *Sclerophthora rayissiae*, *Sclerospora graminicola*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora sacchari*, *Peronosclerospora maydis*; rusts such as *Puccinia sorphi*, *Puccinia polysora*, *Physopella zae*, etc.; and to other fungal diseases e.g. *Cercospora zae-maydis*, *Colletotrichum graminicola*, *Fusarium monoliforme*, *Exserohilum turcicum*, *Bipolaris maydis*, *Phytophthora parasitica*, *Peronospora tabacina*, *Septoria*, etc.; resistance to viruses or viroids e.g. to tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato bushy stunt virus, and like viruses; rrResistance to insects, such as to aphids e.g. *Myzus persicae*; to beetles and beetle larvae; to lepidoptera larvae, e.g. *Heliothis* etc.; resistance to Nematodes, e.g. *Meloidogyne incognita* etc.; local resistance in primary (infected) or secondary (uninfected) leaves; stress tolerance; winter survival; cold tolerance; salt tolerance; heavy metal tolerance, such as cadmium; tolerance to physical wounding; increased organelle tolerance to redox stress, such as in mitochondria, and chloroplasts; cell death; programmed cell death, including death of diseased tissue and during senescence; fruit drop; biomass; fresh and dry weight during any time in plant life, such as maturation; number of flowers, seeds, branches, and/or leaves; seed yield, including number, size, weight, and/or harvest index; fruit yield, including number, size, weight, and/or harvest index; plant development; time to fruit maturity; cell wall strengthening and reinforcement; plant product quality; paper making quality; food additives; treatment of indications modulated by free radicals; cancer; kinds of low molecular weight compounds such as phytoalexins; abundance of low molecular weight compounds such as phytoalexins; other phenotypes based on gene silencing.

To regulate any of the phenotype(s) above, activities of one or more of the disease responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in Winkler et al. (1998) *Plant Physiol* 118: 743-50 and assayed , for example, in accordance to Alvarez et al., (1998) *Cell* 92: 773-784; Halbrock and Scheel; (1989) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 40: 347-369; Lamb et al., (1997) *Ann. Rev. Plant Mol. Biol. Plant Physiol.* 48: 251-275;

Lapwood et al. (1984) Plant Pathol. 33: 13-20; Levine et al. (1996) Curr. Biol. 6: 427-437;  
McKersie et al., (2000) Plant Physiol. 122: 1427-1437; Olson and Varner (1993) Plant J. 4: 887-  
892; Pastore et al., (2000), FEBS Lett 470: 88-92; Pastori et al., (1997) Plant Physiol. 113: 411-418;  
Romero-Puertas et al., (1999) Free Radic. Res. 1999 31 Suppl: S25-31; Shirataki et al., Anticancer  
Res 20: 423-426 (2000); Wu et al., (1995) Plant Cell 7: 1357-1368.

III.E.12.b. Use Of Disease Responsive Genes, Gene Components And  
Products To Modulate Biochemical Activities

The activities of one or more of the disease responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations above and included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Resistance to Pathogens	Induction of ROS signaling pathways	Wu et.al.(1995) Plant Cell 7: 1357-68
	Modulation of nitric oxide signaling	Delledonne et al. (1998) Nature 394: 585-588
	Induction of PR proteins, phytoalexins, and defense pathways	Chamnongpol et.al.(1998) Proc. Nat.Acad Sci USA 12;95:5818-23. Davis et al. (1993) Phytochemistry 32: 607-611

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Induction of cellular protectant genes such as glutathione S-transferase (GST) and ascorbate peroxidase	Chen et.al. Plant J. (1996) 10:955-966 Gadea et.al.(1999) Mol Gen Genet 262:212-219 Wu et.al.(1995) Plant Cell 7: 1357-68
	ROS levels following wounding and changes in physical pressure	Orozco-Cardenas and Ryan (1999) Proc.Nat. Acad. Sci. USA 25;96:6553-7. Yahraus et al. (1995) Plant Physiol. 109: 1259-1266
	Salicyclic acid levels and signaling	Durner and Klessig (1996) J.Biol.Chem. 271:28492-501
Responses to Wounding	Expression of genes Involved in wound repair and cell division	Legendre et al. (1993) Plant Physiol. 102: 233-240
Responses to Environmental Stress	Expression of genes involved in responses to drought, cold, salt, heavy metals	Shi et al. (2000) Proc. Natl. Acad. Sci. USA 97:6896-6901
Reinforcement of Cell Walls	Modulation of the Production of ExtracTable Proline-Rich Protein	Bradley et al. (1992) Cell 70, 21-30
	Modulation of Lignification	Mansouri et al. (1999) Physiol. Plant 106: 355-362

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Programmed Cell Death	Induction of PCD activating genes	Levine et al. (1996) Curr. Biol. 6: 427-437. Reynolds et.al. (1998) Biochem.J. 330:115-20
	Suppression of PCD suppressing genes	Pennell and Lamb (1997) Plant Cell 9, 1157-1168

Other biological activities that can be modulated by the disease responsive genes and their products are listed in the Reference Table. Assays for detecting such biological activities are described in the Protein Domain table.

Disease responsive genes are characteristically differentially transcribed in response to fluctuating levels of disease. The MA\_diff table(s)report(s) the changes in transcript levels of various disease responsive genes in the aerial parts of a plant 3 days after the plant was sprayed with a suspension of TMV relative to control plants sprayed with water.

The data from this experiment reveal a number of types of disease responsive genes and gene products, including "early responders," and "delayed responders". Profiles of individual disease responsive genes are shown in the Table below with examples of which associated biological activities are modulated when the activities of one or more such genes vary in plants.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
Upregulated transcripts	Early Responders to Pathogens	ROS Perception and Response	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat

			proteins (LRRs), transporters, calcium binding proteins, chromatin remodeling proteins
		Initiation of Gene Transcription	Glutathione S-transferase (GST), heat shock proteins, salicylic acid (SA) response pathway proteins, jasmonate response pathway proteins, dehydrins, peroxidases, catalases
	Delayed Responders to Pathogens	Initiation of Defence Gene Transcription	Proteases, pathogen response (PR) proteins, cellulases, chitinases, cutinases, glucanases, other degrading enzymes, calcium channel blockers, phenylalanine ammonia lyase, proteins of defense pathways, cell wall proteins including proline rich proteins and glycine rich proteins, epoxide hydrolase, methyl transferases
		Activation of cell death pathways	Transcription factors kinases, phosphatases, DNA surveillance proteins, p53, proteases, endonucleases,

			GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, mitochondrial and chloroplast energy related proteins, ribosome inactivating proteins
		Initiation of Cellular Protectant Gene Transcription	Reactive oxygen scavenging enzymes, GST, catalase, peroxidase, ascorbate oxidase
Downregulated transcripts	Early responders to pathogens	Negative regulation of pathogen inducible pathways released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins
	Genes repressed by TMV	Negative regulation of ROS inducible pathways released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin

			remodelling proteins
	Delayed Responders to Pathogens	Negative regulation of pathogen inducible pathways released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins
	Genes repressed by TMV	Negative regulation of genes suppressing programmed cell death released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins

#### USE OF PROMOTERS OF DISEASE RESPONSIVE GENES

Promoters of Disease responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Disease responsive genes where the desired sequence is operably linked to a promoter of a Disease responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **II.E.13. DEFENSE (LOL2) RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Often growth and yield are limited by the ability of a plant to tolerate stress conditions, including pathogen attack. To combat such conditions, plant cells deploy a battery of inducible defense responses, including the triggering of an oxidative burst and the transcription of pathogenesis-related protein (PR protein) genes. Reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> and NO from the oxidative burst play a signaling role, including initiation of the hypersensitive response (HR) and induction of systemic acquired resistance (SAR) to secondary infection by unrelated pathogens. Some PR proteins are able to degrade the cell walls of invading microorganisms, and phytoalexins are directly microbicidal. Other defense related pathways are regulated by salicylic acid (SA) or methyl jasmonate (MeJ).

These responses depend on the recognition of a microbial avirulence gene product (avr) by a plant resistance gene product (R), and a series of downstream signaling events leading to transcription-independent and transcription-dependent disease resistance responses. Current models suggest that R-gene-encoded receptors specifically interact with pathogen-encoded ligands to trigger a signal transduction cascade. Several components include *ndr1* and *eds1* loci. *NDR1*, *EDS1*, *PR1*, as well as *PDF1.2*, a MeJ regulated gene and *Nim1*, a SA regulated gene, are differentially regulated in plants with mutations in the *LOL2* gene.

*LOL2* shares a novel zinc finger motif with *LSD1*, a negative regulator of cell death and defense response. Due to an alternative splice site the *LOL2* gene encodes two different proteins, one of which contains an additional, putative DNA binding motif. Northern analysis demonstrated that *LOL2* transcripts containing the additional DNA binding motif are predominantly upregulated after treatment with both virulent and avirulent *Pseudomonas syringae* pv *maculicola* strains. Modulation in this gene can also confer enhanced resistance to virulent and avirulent *Peronospora parasitica* isolates.

Examples of *LOL2* responsive genes and gene products are shown in the Reference, Sequence, Protein Group, Protein Group Matrix, *MA\_diff* and *MA\_clust* tables. These genes and/or products are responsible for effects on traits such as plant vigor, disease resistance, and seed yield. The genes were discovered and characterized from a much larger set by microarray experiments designed to find genes whose mRNA products changed when the *LOL2* gene was mutated in plants.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing some about 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in plants with the LOL2 mutation versus wildtype were obtained. Specifically, the plant line lol-2-2 tested, a loss of function mutation. The ESTs were compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. The MA\_diff table reports the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which represent LOL2 responsive genes.

Manipulation of one or more LOL2 responsive gene activities is useful to modulate the biological processes and/or phenotypes listed below. LOL2 responsive genes and gene products can act alone or in combination. Useful combinations include LOL2 responsive genes and/or gene products with similar transcription profiles, similar biological activities, or members of the same or functionally related biochemical pathways. Whole pathways or segments of pathways are controlled by transcription factor proteins and proteins controlling the activity of signal transduction pathways. Therefore, manipulation of such protein levels is especially useful for altering phenotypes and biochemical activities of plants.

Such LOL2 responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in active LOL2 gene or in the absence. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: lol2 (relating to SMD 8031, SMD 8032)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Defense genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

Defense Genes Identified By Cluster Analyses Of Differential Expression

Defense Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Defense genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID lol2 (relating to SMD 8031, SMD 8032) of the MA\_diff table(s).

Defense Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Defense genes. A group in the MA\_clust is considered a Defense pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Defense Genes Identified By Amino Acid Sequence Similarity

Defense genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Defense genes. Groups of Defense genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Defense pathway or network is a group of proteins that also exhibits Defense functions/utilities.

Further, promoters of LOL2 responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by LOL2 responsive genes or any of the following phenotypes or biological activities below. Further, any desired sequence can be

transcribed in similar temporal, tissue, or environmentally specific patterns as the LOL2 responsive genes when the desired sequence is operably linked to a promoter of a LOL2 responsive gene.

III.E.12.a. Use Of Lol2 Responsive Genes, Gene Components And Products To Modulate Phenotypes

LOL2 responsive genes and gene products are useful to or modulate one or more phenotypes including pathogen tolerance and/or resistance; Avr/r locus interactions; Non-Host interactions; HR; SAR, e.g., disease responsive genes acting in conjunction with infection with any of the organisms listed below; resistance to bacteria e.g. to *Erwinia stewartii*, *Pseudomonas syringae*, *Pseudomonas tabaci*, Stuart's wilt, etc.; resistance to fungi e.g. to downy mildews such as *Sclerophthora macrospora*, *Sclerophthora rayissiae*, *Sclerospora graminicola*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Peronosclerospora sacchari*, *Peronosclerospora maydis*; rusts such as *Puccinia sorphi*, *Puccinia polysora*, *Physopella zae*, etc.; and to other fungal diseases e.g. *Cercospora zeae-maydis*, *Colletotrichum graminicola*, *Fusarium monoliforme*, *Exserohilum turcicum*, *Bipolaris maydis*, *Phytophthora parasitica*, *Peronospora tabacina*, *Septoria*, etc.; resistance to viruses or viroids e.g. to tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, pelargonium leaf curl virus, red clover mottle virus, tomato bushy stunt virus, and like viruses; resistance to insects, such as to aphids e.g. *Myzus persicae*; to beetles and beetle larvae; to lepidoptera larvae, e.g. *Heliothis* etc.; resistance to nematodes, e.g. *Meloidogyne incognita* etc.; local resistance in primary (infected) or secondary (uninfected) leaves; stress tolerance; winter survival; cold tolerance; salt tolerance, heavy metal tolerance, such as cadmium; tolerance to physical wounding; increased organelle tolerance to redox stress, such as in mitochondria, and chloroplasts; cell death; programmed cell death, including death of diseased tissue and during senescence; fruit drop; biomass; fresh and dry weight during any time in plant life, such as maturation; number of flowers, seeds, branches, and/or leaves; seed yield, including number, size, weight, and/or harvest index; fruit yield, including number, size, weight, and/or harvest index; plant development; time to fruit maturity; cell wall strengthening and reinforcement; plant product quality; paper making quality; food additives; treatment of indications modulated by free radicals; cancer; kinds of low molecular

weight compounds such as phytoalexins; abundance of low molecular weight compounds such as phytoalexins; and other phenotypes based on gene silencing.

To regulate any of the phenotype(s) above, activities of one or more of the LOL2 responsive genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and assayed , for example, in accordance to Alvarez et al., (1998) Cell 92: 773-784; Halbrook and Scheel, (1989) Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 347-369; Lamb et al., (1997) Ann. Rev. Plant Mol. Biol. Plant Physiol. 48: 251-275; Lapwood et al. (1984) Plant Pathol. 33: 13-20; Levine et al. (1996) Curr. Biol. 6: 427-437; McKersie et al., (2000) Plant Physiol. 122: 1427-1437; Olson and Varner (1993) Plant J. 4: 887-892; Pastore et al., (2000), FEBS Lett 470: 88-92; Pastori et al., (1997) Plant Physiol. 113: 411-418; Romero-Puertas et al., (1999) Free Radic. Res. 1999 31 Suppl: S25-31; Shirataki et al., Anticancer Res 20: 423-426 (2000); Wu et al., (1995) Plant Cell 7: 1357-1368.

III.E.12.b. Use Of Defense Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the defense (LOL2) responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities are documented and can be measured according to the citations above and included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Resistance To Pathogens	Induction Of ROS Signaling Pathways	Wu et.al.(1995) Plant Cell 7: 1357-68
	Modulation Of Nitric Oxide Signaling	Delledonne et al. (1998) Nature 394: 585-588

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Induction Of PR Proteins, Phytoalexins, And Defense Pathways	Chamnongpol et.al.(1998) Proc. Nat.Acad Sci USA 12;95:5818-23. Davis et al. (1993) Phytochemistry 32: 607-611
	Induction Of Cellular Protectant Genes Such As Glutathione S-Transferase (GST) And Ascorbate Peroxidase	Chen et.al. Plant J. (1996) 10:955-966 Gadea et.al.(1999) Mol Gen Genet 262:212-219 Wu et.al.(1995) Plant Cell 7: 1357-68
	ROS Levels Following Wounding And Changes In Physical Pressure	Orozco-Cardenas and Ryan (1999) Proc.Nat. Acad. Sci. USA 25;96:6553-7. Yahraus et al. (1995) Plant Physiol. 109: 1259-1266
	Salicyclic Acid Levels And Signaling	Durner and Klessig (1996) J.Biol.Chem. 271:28492-501
Responses To Wounding	Expression Of Genes Involved In Wound Repair And Cell Division	Legendre et al. (1993) Plant Physiol. 102: 233-240
Responses To Environmental Stress	Expression Of Genes Involved In Responses To Drought, Cold, Salt, Heavy Metals	Shi et al. (2000) Proc. Natl. Acad. Sci. USA 97:6896-6901

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Reinforcement Of Cell Walls	Modulation Of The Production Of ExtracTable Proline-Rich Protein	Bradley et al. (1992) Cell 70, 21-30
	Modulation Of Lignification	Mansouri et al. (1999) Physiol. Plant 106: 355-362
Programmed Cell Death	Induction Of Pcd Activating Genes	Levine et al. (1996) Curr. Biol. 6: 427-437. Reynolds et.al. (1998) Biochem.J. 330:115-20
	Suppression Of PCD Suppressing Genes	Pennell and Lamb (1997) Plant Cell 9, 1157-1168

Other biological activities that can be modulated by the LOL2 responsive genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

LOL2 responsive genes are characteristically differentially transcribed in response to fluctuating levels of disease. MA\_diff table reports the changes in transcript levels of various LOL2 responsive genes in the lol-2 line versus control plants.

The data from this experiment reveal a number of types of LOL2 responsive genes and gene products. Profiles of individual LOL2 responsive genes are shown in the Table below with examples of which associated biological activities are modulated when the activities of one or more such genes vary in plants.

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSequence	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
Upregulated transcripts	Early Responders to the LOL2 Mutation	ROS Perception and Response	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodeling proteins
		Initiation of Gene Transcription	Glutathione S-transferase (GST), heat shock proteins, salicylic acid (SA) response pathway proteins, jasmonate response pathway proteins, dehydrins, peroxidases, catalases
	Delayed Responders to the LOL2 Mutation	Initiation of Defence Gene Transcription	Proteases, pathogen response (PR) proteins, cellulases, chitinases, cutinases, glucanases, other degrading enzymes, calcium channel blockers, phenylalanine ammonia lyase, proteins of defense pathways, cell wall proteins

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
			including proline rich proteins and glycine rich proteins, epoxide hydrolase, methyl transferases
		Activation of cell death pathways	Transcription factors kinases, phosphatases, DNA surveillance proteins, p53, proteases, endonucleases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, mitochondrial and chloroplast energy related proteins, ribosome inactivating proteins
		Initiation of Cellular Protectant Gene Transcription	Reactive oxygen scavenging enzymes, GST, catalase, peroxidase, ascorbate oxidase
Downregulated transcripts	Early Responders to the LOL2	Negative regulation of LOL2 Mutation inducible pathways	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
	Mutation	released	proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins
	Genes Repressed by the LOL2 Mutation	Negative regulation of ROS inducible pathways released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins
	Delayed Responders to the LOL2 Mutation	Negative regulation of LOL2 Mutation inducible pathways released	Transcription factors, kinases, phosphatases, GTP-binding proteins (G-proteins), leucine rich repeat proteins (LRRs), transporters, calcium binding proteins, chromatin remodelling proteins
	Genes Repressed By The LOL2 Mutation	Negative Regulation Of Genes Suppressing Programmed Cell Death Released	Transcription Factors, Kinases, Phosphatases, GTP-Binding Proteins (G-Proteins), Leucine Rich

GENE EXPRESSION LEVELS	FUNCTIONAL CATEGORY OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY OF GENE PRODUCTS
			Repeat Proteins (Lrrs), Transporters, Calcium Binding Proteins, Chromatin Remodelling Proteins

#### USE OF PROMOTERS OF DEFENSE RESPONSIVE GENES

Promoters of Defense responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Defense responsive genes where the desired sequence is operably linked to a promoter of a Defense responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.E.14. IRON RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Iron (Fe) deficiency in humans is the most prevalent nutritional problem worldwide today. Increasing iron availability via diet is a sustainable malnutrition solution for many of the world's nations. One-third of the world's soils, however, are iron deficient. Consequently, to form a food-based solution to iron malnutrition, we need a better understanding of iron uptake, storage and utilization by plants. Furthermore, exposure to non-toxic Fe levels appears to affect inherent plant defense mechanisms. Consequently, exploring the effects of Fe exposure has potential for advances in plant disease resistance in addition to human nutrition.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent FeNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing 10,000 non-redundant ESTs, selected from 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full length FeNA and genomic sequence databanks, and identical Ceres clones identified. MA\_diff table reports the results of this analysis, indicating those Ceres clones that are up or down regulated over controls, thereby indicating the Ceres clones which are iron responsive genes.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Iron (relating to SMD 7114, SMD 7115, SMD 7125)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Iron genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Iron Genes Identified By Cluster Analyses Of Differential Expression

#### Iron Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Iron genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Iron (relating to SMD 7114, SMD 7115, SMD 7125) of the MA\_diff table(s).

Iron Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Iron genes. A group in the MA\_clust is considered a Iron pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Iron Genes Identified By Amino Acid Sequence Similarity

Iron genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Iron genes. Groups of Iron genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Iron pathway or network is a group of proteins that also exhibits Iron functions/utilities.

III.E.14.a. Use Of Iron Responsive Genes To Modulate Phenotypes

Iron responsive genes and gene products are useful to or modulate one or more phenotypes including growth; roots; root hair formation; stems, leaves; development; senescence; plant nutrition; uptake and assimilation of organic compounds; uptake and assimilation of inorganic compounds; animal (including human) nutrition; improved dietary mineral nutrition; stress response metabolic detoxification; and heavy metals.

To improve any of the phenotype(s) above, activities of one or more of the iron responsive genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Schmidt et al. (2000, Plant Physiol 122:1109-18), Meagher (2000) Current Opinion

in Plant Biology 3: 153-62), Deak (1999, Nature Biotechnology (1999, Nature Biotechnology 17: 192-96), Wei and Theil (2000, J. Biol Chem 275: 17488-93) and Vansuyt et al. (1997, FEBS Letters 410: 195-200).

III.E.14.b. Use Of Iron-Responsive Genes, Gene Components And Products To Modulate Biochemical Activities

The activities of one or more of the iron responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth , Differentiation and Development	<ul style="list-style-type: none"><li>• Root Growth<ul style="list-style-type: none"><li>- Initiation of root hairs</li></ul></li></ul>	Robinson et al. (1999) Nature 397: 694-97
Metabolisms	<ul style="list-style-type: none"><li>• Iron sensing</li><li>• Iron uptake and transport<ul style="list-style-type: none"><li>-decreased iron transport</li><li>-phytoremediation</li></ul></li></ul>	Thomine et al. (2000) PNAS USA 97: 4991-6 Thomine et al. (2000) PNAS USA 97: 4991-6 Zhu (1999) Plant Physiol 119: 73-79
Plant Defenses	<ul style="list-style-type: none"><li>• Protection from oxidative damage</li></ul>	Deak (1999) Nature Biotechnology 17: 192-6
Signaling	<ul style="list-style-type: none"><li>• Specific gene transcription gene silencing</li></ul>	Brand and Perrimon (1993) Development 118: 401-415

Other biological activities that can be modulated by the iron responsive genes and gene products are listed in the REFERENCE Table. Assays for detecting such biological activities are described in the Protein Domain table.

Iron responsive genes are characteristically differentially transcribed in response to fluctuating iron levels or concentrations, whether internal or external to an organism or cell. MA\_diff table reports the changes in transcript levels of various iron responsive genes.

The microarray comparison consists of probes prepared from root RNA of A. thaliana (Columbia) seedlings grown under iron-sufficient conditions and seedlings grown under iron-deficient. The data from this experiment reveal a number of types genes and gene products. Profiles of these different iron responsive genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	responders to iron application	<ul style="list-style-type: none"><li>• Iron perception</li><li>• Iron uptake and transport</li><li>• Iron metabolism</li><li>• Synthesis of secondary metabolites and/or proteins</li><li>• Modulation of iron response transduction pathways</li></ul>	<ul style="list-style-type: none"><li>• Transporters</li><li>• Metabolic enzymes</li><li>• Change in cell membrane structure and potential</li><li>• Kinases and phosphatases</li><li>• Transcription activators</li><li>• Change in chromatin structure and/or localized DNA topology</li></ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>Specific gene transcription initiation</li> </ul>	
Down-regulated transcripts	<p>responder to iron repressors of iron state of metabolism</p> <p>Genes with discontinued expression or unsTable mRNA in presence of iron</p>	<ul style="list-style-type: none"> <li>Negative regulation of iron pathways</li> <li>Changes in pathways and processes operating in cells</li> <li>Changes in other metabolisms than iron</li> </ul>	<ul style="list-style-type: none"> <li>Transcription factors</li> <li>Change in protein structure by phosphorylation (kinases) or dephosphoryaltion (phosphatases)</li> <li>Change in chromatin structure and/or DNA topology</li> <li>Stability of factors for protein synthesis and degradation</li> <li>Metabolic enzymes</li> </ul>

#### USE OF PROMOTERS OF IRON RESPONSIVE GENES

Promoters of Iron responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Iron responsive genes where the desired sequence is operably linked to a promoter of a Iron responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such

promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### III.E.15. SHADE RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS

Plants sense the ratio of Red (R) : Far Red (FR) light in their environment and respond differently to particular ratios. A low R:FR ratio, for example, enhances cell elongation and favors flowering over leaf production. The changes in R:FR ratios mimic and cause the shading response effects in plants. The response of a plant to shade in the canopy structures of agricultural crop fields influences crop yields significantly. Therefore manipulation of genes regulating the shade avoidance responses can improve crop yields. While phytochromes mediate the shade avoidance response, the down-stream factors participating in this pathway are largely unknown. One potential downstream participant, ATHB-2, is a member of the HD-Zip class of transcription factors and shows a strong and rapid response to changes in the R:FR ratio. ATHB-2 overexpressors have a thinner root mass, smaller and fewer leaves and longer hypocotyls and petioles. This elongation arises from longer epidermal and cortical cells, and a decrease in secondary vascular tissues, paralleling the changes observed in wild-type seedlings grown under conditions simulating canopy shade. On the other hand, plants with reduced ATHB-2 expression have a thick root mass and many larger leaves and shorter hypocotyls and petioles. Here, the changes in the hypocotyl result from shorter epidermal and cortical cells and increased proliferation of vascular tissue. Interestingly, application of Auxin is able to reverse the root phenotypic consequences of high ATHB-2 levels, restoring the wild-type phenotype. Consequently, given that ATHB-2 is tightly regulated by phytochrome, these data suggest that ATHB-2 may link the Auxin and phytochrome pathways in the shade avoidance response pathway.

Changes in R:FR ratios promote changes in gene expression. Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by hybridizing labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The US Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments

conducted with AFGC chips containing about 10,000 non-redundant ESTs, selected from about 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases in plants given 4 hours of FR rich light after growth in high R:FR light compared with the controls of plants grown in high R:FR light only, were identified, compared to the Ceres full length cDNA and genomic sequence databanks, and equivalent Ceres clones identified. The MA\_diff table(s) report(s) the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which are shade avoidance responsive genes.

Examples of far red light induced, shade avoidance responsive genes and gene products are shown in the Reference and Sequence Tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While far red light, shade avoidance responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different shade avoidance responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of a shade avoidance responsive polynucleotide and/or gene product with another environmentally responsive polynucleotides is also useful because of the interactions that exist between hormone-regulated pathways, stress and pathogen induced pathways, nutritional pathways, light induced pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in common or overlapping pathways.

Such far red light induced shade avoidance responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either in response to changes in far red light or in the absence of far red light fluctuations. The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Shade (relating to SMD 8130, SMD 7230)). For transcripts that had higher levels in the samples than the control, a "+" is

shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Shade genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Shade Genes Identified By Cluster Analyses Of Differential Expression

##### Shade Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Shade genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Shade (relating to SMD 8130, SMD 7230) of the MA\_diff table(s).

##### Shade Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Shade genes. A group in the MA\_clust is considered a Shade pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

##### Shade Genes Identified By Amino Acid Sequence Similarity

Shade genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Shade genes. Groups of Shade genes are identified in the Protein Group table. In this table, any protein group that comprises a

peptide ID that corresponds to a cDNA ID member of a Shade pathway or network is a group of proteins that also exhibits Shade functions/utilities.

Further, promoters of shade avoidance responsive genes, as described in the Reference tables, for example, are useful to modulate transcription that is induced by shade avoidance or any of the following phenotypes or biological activities below. Further, any desired sequence can be transcribed in similar temporal, tissue, or environmentally specific patterns as the shade avoidance responsive genes when the desired sequence is operably linked to a promoter of a circadian (clock) responsive gene.

III.E.15.a. Use Of Far Red Responsive, Shade Avoidance Response Genes To Modulate Phenotypes

High FR:R, shade avoidance responsive genes and gene products can be used to alter or modulate one or more phenotypes including growth; roots; elongation; lateral root formation; stems; elongation; expansion; leaves; expansion; carotenoid composition; development; cell; photosynthetic apparatus; efficiency; flower; flowering time; fruit; seed; dormancy; control rate and timing of germination; prolongs seed storage and viability; inhibition of hydrolytic enzyme synthesis; seed and fruit yield; senescence; abscission; leaf fall; flower longevity; differentiation; vascularization; and shade (avoidance) responses in plant architecture.

To regulate any of the phenotype(s) above, activities of one or more of the High FR: R light, shade avoidance responsive genes or gene products can be modulated and the plants tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and/or screened for variants as in Winkler et al. (1998) Plant Physiol 118: 743-50 and visually inspected for the desired phenotype or metabolically and/or functionally assayed according to Carabelli et al. (1996, PNAS USA 93: 3530-3535), Aguirrezabal and Tardieu (1996, J Exp Bot 47: 411-20), Heyer et al. (1995, Plant Physiol 109: 53-61), Garcia-Plazaola et al. (1997, J Exp Bot 48: 1667-74), Schwanz et al. (1996, J Exp Bot 47L 1941-50), Koehne et al. (1999, Biochem Biophys Acta 1412:94-107), Melis (1984, J Cell Biochem 24: 271-85), Steindeler et al. (1999, Development 126: 4235-45), Cruz (1997, J Exp Bot 48: 15-24), Stephanou and Manetas (1997, J

Exp Bot 48: 1977-85), Grammatikopoulos et al (1999, J Exp Bot 50:517-21), Krause et al. (1999, Plant Physiol 121: 1349-58), Aukerman et al. (1997, Plant Cell 9: 1317-26), Wagner et al. (1997, Plant Cell 9: 731-43), Weinig (2000) Evolution Int J Org Evolution 54: 124-26), Cocburn et al. (1996, J Exp Bot 47: 647-53), Devlin et al. (1999, Plant Physiol 119: 909-15), Devlin et al. (1998, Plant Cell 10: 1479-87), Finlayson et al. (1998, Plant Physiol 116: 17-25), Morelli and Ruberti (2000, Plant Physiol 122: 621-26), Aphalo et al. (1999, J Exp Bot 50: 1629-34), Sims et al. (1999, J Exp Bot 50: 50: 645-53) and Ballare (1999, Trends Plant Sci 4: 97-102).

III.E.15.b. Use Of Far Red Light, Shade Avoidance Responsive Genes To Modulate Biochemical Activities

The activities of one or more of the far red light, shade avoidance responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Cell Growth and Differentiation	Cell Elongation	Carabelli et al. (1996) PNAS USA 93: 3530-35
	Leaf Expansion	Heyer et al. (1995) Plant Physiol 109: 53-61
Photosynthesis	Development of Photosynthetic Apparatus	Jagtap et al. (1998) J Exp Bot 49: 1715-21 Melis (1984) J Cell Biochem 24: 271-285 McCain (1995) Biophys J 69: 1105-10

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Carotenoid Composition	Garcia-Plazaola et al (1997) J Exp Bot 48: 1667-74
Carbon/Nitrogen Metabolism	Carbon and Nitrogen Assimilation	Cruz (1997) J Exp Bot 48: 15-24
Far red light, shade avoidance response binding by transcription factors		Newton AL, Sharpe BK, Kwan A, Mackay JP, Crossley M. J Biol Chem. 2000 May 19;275(20):15128-34; Lopez Ribera I, Ruiz-Avila L, Puigdomenech P. Biochem Biophys Res Commun. 1997 Jul 18;236(2):510-6; de Pater S, Greco V, Pham K, Memelink J, Kijne J. Nucleic Acids Res. 1996 Dec 1; 24(23):4624-31.
Signaling	UV Light Perception	Stephanou and Manetas (1997) J Exp Bot 48: 1977-85
	Far-red/Red Light Perception	Aukerman et al. (1997) Plant Cell 9: 1317-26 Wagner et al. (1997) Plant Cell 9: 731-43
	Interaction of "Shade Factor" with Ethylene Production/Transduction	Finlayson et al. (1998) Plant Physiol 116: 17-25

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	Interaction of "Shade Factor" with Auxin Production/Transduction	Reed et al. (1998) Plant Physiol 118: 1369-78
	Plant to Plant signalling	Sims et al. (1999) J Exp Bot 50: 645-53

Other biological activities that can be modulated by shade avoidance response genes and their products are listed in the REF TABLES. Assays for detecting such biological activities are described in the Protein Domain table.

High FR:R, shade avoidance responsive genes are differentially transcribed in response to high FR:R ratios. The microarray comparison to reveal such genes consisted of probes prepared from RNA isolated from the aerial tissues of *A. thaliana* (Columbia) two-week old seedlings grown in high R:FR ratios compared to seedlings grown in high R:FR ratios followed by 4 hours of FR-rich light treatment. The data from this experiment reveal a number of types genes and gene products and examples of the classes of genes are given in the Table below.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	Responders to high FR:R light ratios	<ul style="list-style-type: none"> <li>• Far red light perception</li> <li>• Metabolism affected by far red</li> </ul>	<ul style="list-style-type: none"> <li>• Transporters</li> <li>• Metabolic enzymes</li> <li>• Change in cell membrane structure</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
	Genes induced by high FR:R light ratio	<p>light</p> <ul style="list-style-type: none"> <li>• Synthesis of secondary metabolites and/or proteins</li> <li>• Modulation of high FR:R light ratio transduction pathways</li> <li>• Specific gene transcription initiation</li> </ul>	<p>and potential</p> <ul style="list-style-type: none"> <li>• Kinases and phosphatases</li> <li>• Transcription activators</li> <li>• Change in chromatin structure and/or localized DNA topology</li> <li>• Leaf production factors</li> </ul>
Down-regulated transcripts	<p>Responders to high FR:R light ratios</p> <p>Genes repressed by high FR:R light ratio</p> <p>Genes with discontinued expression or unTable mRNA during high FR:R ratio light</p>	<ul style="list-style-type: none"> <li>• Changes in pathways and processes operating in cells</li> <li>• Changes in metabolisms other than far red stimulated pathways</li> </ul>	<ul style="list-style-type: none"> <li>• Transcription factors</li> <li>• Change in protein structure by phosphorylation (kinases) or dephosphorylation (phosphatases)</li> <li>• Change in chromatin structure and/or DNA topology</li> <li>• Stability of factors for protein synthesis and degradation</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
			<ul style="list-style-type: none"><li>• Metabolic enzymes</li><li>• Cell elongation factors</li><li>• Flowering promotion factors</li></ul>

#### USE OF PROMOTERS OF SHADE AVOIDANCE GENES

Promoters of Shade Avoidance genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Shade Avoidance genes where the desired sequence is operably linked to a promoter of a Shade Avoidance gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### **III.E.16. SULFUR RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Sulfur is one of the important macronutrients required by plants. It is taken up from the soil solution by roots as in the form of sulfate anion which higher plants are dependent on to fulfill their nutritional sulfur requirement. After uptake from the soil, sulfate is either accumulated and stored in vacuole or it is assimilated into various organic compounds, e.g. cysteine, glutathione, methionine, etc. Thus, plants also serve as nutritional sulfur sources for animals. Sulfur can be assimilated in one of two ways: it is either incorporated as sulfate in a reaction called sulfation, or it is first reduced to sulfide, the substrate for cysteine synthesis. In plants, majority of sulfur is assimilated in reduced form.

Sulfur comprises a small by vital fraction of the atoms in many protein molecules. As disulfide bridges, the sulfur atoms aid in stabilizing the folded proteins, such cysteine residues.

Cys is the first sulfur-containing amino acids, which in proteins form disulfide bonds that may affect the tertiary structures and enzyme activities. This redox balance is mediated by the disulfide/thiol interchange of thioredoxin or glutaredoxin using NADPH as an electron donor. Sulfur can also become sulfhydryl (SH) groups participating in the active sites of some enzymes and some enzymes require the aid of small molecules that contain sulfur. In addition, the machinery of photosynthesis includes some sulfur-containing compounds, such as ferrodoxin.. Thus, sulfate assimilation plays important roles not only in the sulfur nutrition but also in the ubiquitous process that may regulate the biochemical reactions of various metabolic pathways.

Deficiency of sulfur leads to a marked chlorosis in younger leaves, which may become white in color. Other symptoms of sulfur deficiency also include weak stems and reduced growth. Adding sulfur fertilizer to plants can increase root development and a deeper green color of the leaves in sulfur-deficient plants. However, Sulfur is generally sufficient in soils for two reasons: it is a contaminant in potassium and other fertilizers and a product of industrial combustion. Sulfur limitation in plants is thus likely due to the limitation of the uptake and distribution of sulfate in plants. Seven cell type specific sulfate transporter genes have been isolated from *Arabidopsis*. In sulfate-starved plants, expression of the high-affinity transporter, AtST1-1, is induced in root epidermis and cortex for acquisition of sulfur. The low affinity transporter, AtST2-1 (AST68), accumulates in the root vascular tissue by sulfate starvation for root-to-shoot transport of sulfate. These studies have shown that the whole-plant process of sulfate transport is coordinately regulated by the expression of these 2 sulfate transporter genes under sulfur limited conditions. Recent studies have proposed that feeding of O-acetylserine, GSH and selenate may regulate the expression of AtST1-1 and AtST2-1 (AST68) in roots either positively or negatively. However, regulatory proteins that may directly control the expression of these genes have not been identified yet.

It has been established that there are regulatory interactions between assimilatory sulfate and nitrate reduction in plants. The two assimilatory pathways are very similar and well coordinated; deficiency for one element was shown to repress the other pathway. The coordination between them should be taken into consideration when one tries to alter one of pathways.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The Arabidopsis Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing 10,000 non-redundant ESTs, selected from 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full-length cDNA and genomic sequence databanks, and identical Ceres clones identified. MA\_diff table reports the results of this analysis, indicating those Ceres clones which are up or down regulated over controls, thereby indicating the Ceres clones which are sulfur response responsive genes.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Sulfur (relating to SMD 8034, SMD 8035)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-“ is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Sulfur genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Sulfur Genes Identified By Cluster Analyses Of Differential Expression

#### Sulfur Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Sulfur genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Sulfur (relating to SMD 8034, SMD 8035) of the MA\_diff table(s).

Sulfur Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Sulfur genes. A group in the MA\_clust is considered a Sulfur pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Sulfur Genes Identified By Amino Acid Sequence Similarity

Sulfur genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Sulfur genes. Groups of Sulfur genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Sulfur pathway or network is a group of proteins that also exhibits Sulfur functions/utilities.

III.E.16.a. Use Of Sulfur Responsive Genes To Modulate Phenotypes

Sulfur responsive genes and gene products are useful to or modulate one or more phenotypes including growth; roots; stems; leaves; development; chloroplasts and mitochondria; fruit development; seed development; seed storage proteins; senescence; differentiation; plastid/chloroplast and mitochondria differentiation; protection against oxidative damage; regulation of enzymes via redox control by thiol groups; metabolic detoxification; photosynthesis; and carbon dioxide fixation.

To improve any of the phenotype(s) above, activities of one or more of the sulfur responsive genes or gene products can be modulated and tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed. As an example, a plant can be transformed according to Bechtold and Pelletier (1998, Methods. Mol. Biol. 82:259-266) and visually inspected for the desired phenotype or metabolically and/or functionally assayed.

according to Saito et al. (1994, Plant Physiol. 106: 887-95), Takahashi et al (1997, Proc. Natl. Acad. Sci. USA 94: 11102-07) and Koprivova et al. (2000, Plant Physiol. 122: 737-46).

III.E.16.b. Use Of Sulfur-Responsive Genes, Gene Components And Products To Modulate Biochemical Activities

The activities of one or more of the sulfur responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Growth , Differentiation and Development	<ul style="list-style-type: none"><li>• Root</li><li>• Leaf</li><li>• Stem</li><li>• Chloroplast/Mitochondria development/differentiation</li><li>• Seed storage protein synthesis</li></ul>	<p>Klein and Klein (1988) Mineral Nutrition, In CM Wilson and J Gregory, eds Fundamentals of Plant Science. Harper and Row Publishers, Inc., NY, p163</p> <p>Rost et al. (1984) The Absorption and Transport System, In R Bem, ed, Botany-A Brief Introduction to Plant Biology. John Wiley and Sons, NY, p96.</p> <p>Huluigue et al. (2000) Biochem Biophys Res Commun 271: 380-5</p> <p>Kapazoglou et al. (2000) Eur J Biochem 267: 352-60</p> <p>Kim et al. (1999) 209: 282-9</p>
Metabolisms	<ul style="list-style-type: none"><li>• Sulfate uptake and transport</li></ul>	Takahashi et al. (1997) Proc Natl

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	<ul style="list-style-type: none"> <li>• Cysteine Biosynthesis</li> <li>• Methionine biosynthesis</li> <li>• Carbon dioxide fixation in photosynthesis</li> <li>• Thioredoxin reduction</li> <li>• Nitrogen metabolism</li> </ul>	<p>Acad Sci USA 94: 11102-07</p> <p>Saito et al. (1992) Proc Natl Acad Sci USA 89: 8078-82</p> <p>Hesse et al. (1999) Amino Acids 16: 113-31</p> <p>Bourgis et al. (1999) Plant Cell 11: 1485-98</p> <p>Buchana (1991) Arch Biochem Biophys 288: 1-9</p> <p>Leustek and Saito (1999) Plant Physiol 120: 637-43</p> <p>Mamedova et al. (1999) FEBS Lett 462: 421-4</p> <p>Koprivova et al. (2000) Plant Physiol. 122: 737-46</p> <p>Yamaguchi et al. (1999) Biosci Biotechnol Biochem 63: 762-6</p>
Plant Defenses	<ul style="list-style-type: none"> <li>• Reduction of oxidative stress – oxygen metabolism and reactive oxygen species</li> <li>• Detoxification of toxins, xenobiotics and heavy metals</li> <li>• Defense against pathogens or microbes</li> <li>• Disease prevention by</li> </ul>	<p>May et al. (1998) J Expt Bio 49: 649-67</p> <p>Kreuz et al. (1996) Plant Physiol 111: 349-53</p> <p>Zhao et al. (1998) Plant Cell 10: 359-70</p> <p>Kyung and Fleming (1997) J Food Prot 60: 67-71</p> <p>Fahey et al. (1997) Proc Natl Acad</p>

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
	<ul style="list-style-type: none"> <li>secondary sulfur-containing compounds</li> <li>Activation of kinases and phosphatases</li> </ul>	<p>Sci USA 94: 10367-72</p> <p>Davis et al. (1999) Plant Cell 11: 1179-90</p>

Other biological activities that can be modulated by the sulfur responsive genes and gene products are listed in the REFERENCE Table. Assays for detecting such biological activities are described in the Protein Domain table.

Sulfur responsive genes are characteristically differentially transcribed in response to fluctuating sulfur levels or concentrations, whether internal or external to an organism or cell.

MA\_diff table reports the changes in transcript levels of various sulfur responsive genes.

Profiles of these different sulfur responsive genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
Up regulated transcripts	<ul style="list-style-type: none"> <li>Responders to sulfur application</li> </ul>	<ul style="list-style-type: none"> <li>Sulfur perception</li> <li>Sulfur uptake and transport</li> <li>Sulfur metabolism</li> <li>Synthesis of secondary metabolites and/or proteins</li> </ul>	<ul style="list-style-type: none"> <li>Transporters</li> <li>Metabolic enzymes</li> <li>Change in cell membrane structure and potential</li> <li>Kinases and phosphatases</li> <li>Transcription activators</li> </ul>

TRANSCRIPT LEVELS	TYPE OF GENES	PHYSIOLOGICAL CONSEQUENCES	EXAMPLES OF BIOCHEMICAL ACTIVITY
		<ul style="list-style-type: none"> <li>Modulation of sulfur response transduction pathways</li> <li>Specific gene transcription initiation</li> </ul>	<ul style="list-style-type: none"> <li>Change in chromatin structure and/or localized DNA topology</li> <li>Redox control</li> </ul>
Down-regulated transcripts	<p>responder to sulfur repressors of sulfur state of metabolism</p> <p>Genes with discontinued expression or unsTable mRNA in presence of sulfur</p>	<ul style="list-style-type: none"> <li>Negative regulation of sulfur pathways</li> <li>Changes in pathways and processes operating in cells</li> <li>Changes in other metabolisms than sulfur</li> </ul>	<ul style="list-style-type: none"> <li>Transcription factors</li> <li>Change in protein structure by phosphorylation (kinases) or dephosphoryaltion (phosphatases)</li> <li>Change in chromatin structure and/or DNA topology</li> <li>Stability of factors for protein synthesis and degradation</li> <li>Metabolic enzymes</li> </ul>

#### USE OF PROMOTERS OF SULFUR RESPONSIVE GENES

Promoters of Sulfur responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Sulfur responsive genes

where the desired sequence is operably linked to a promoter of a Sulfur responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoter are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

### **III.E.17. ZINC RESPONSIVE GENES, GENE COMPONENTS AND PRODUCTS**

Phytoremediation of soils contaminated with toxic levels of heavy metals requires the understanding of plant metal transport and tolerance. The numerous *Arabidopsis thaliana* studies have given scientists the potential for dissection and elucidation of plant micronutrient/heavy metal uptake and accumulation pathways. It has been shown altered regulation of ZNT1, a Zn/Cd transporter, contributes to high Zn uptake. Isolation and characterization of Zn/Cd hyperaccumulation genes may allow expression in higher biomass plant species for efficient contaminated soil clean up. Identification of additional Zn transport, tolerance and nutrition-related genes involved in heavy metal accumulation will enable manipulation of increased uptake (for phytoremediation) as well as limitation of uptake or leak pathways that contribute to toxicity in crop plants. Additionally, Zn-binding ligands involved in Zn homeostasis or tolerance may be identified, as well as factors affecting the activity or expression of Zn binding transcription factors. Gene products acting in concert to effect Zn uptake, which would not have been identified in complementation experiments, including multimeric transporter proteins, could also be identified.

Microarray technology allows monitoring of gene expression levels for thousands of genes in a single experiment. This is achieved by simultaneously hybridizing two differentially labeled fluorescent cDNA pools to glass slides that contain spots of DNA (Schena et al. (1995) Science 270: 467-70). The *Arabidopsis* Functional Genomics Consortium (AFGC) has recently made public the results from such microarray experiments conducted with AFGC chips containing 10,000 non-redundant ESTs, selected from 37,000 randomly sequenced ESTs generated from mRNA of different tissues and developmental stages.

The sequences of the ESTs showing at least two-fold increases or decreases over the controls were identified, compared to the Ceres full-length cDNA and genomic sequence databanks, and identical Ceres clones identified. The Zn response information was then used in conjunction with the existing annotation to attribute biological function or utility to the full-length cDNA and corresponding genomic sequence.

The MA\_diff Table(s) reports the transcript levels of the experiment (see EXPT ID: Zinc (relating to SMD 7310, SMD 7311)). For transcripts that had higher levels in the samples than the control, a “+” is shown. A “-” is shown for when transcript levels were reduced in root tips as compared to the control. For more experimental detail see the Example section below.

Zinc genes are those sequences that showed differential expression as compared to controls, namely those sequences identified in the MA\_diff tables with a “+” or “-“ indication.

#### Zinc Genes Identified By Cluster Analyses Of Differential Expression

#### Zinc Genes Identified By Correlation To Genes That Are Differentially Expressed

As described above, the transcription profiles of genes that act together are well correlated. Applicants not only have identified the genes that are differentially expressed in the microarray experiments, but also have identified the genes that act in concert with them. The MA\_clust table indicates groups of genes that have well correlated transcription profiles and therefore participate in the same pathway or network.

A pathway or network of Zinc genes is any group in the MA\_clust that comprises a cDNA ID that also appears in Expt ID Zinc (relating to SMD 7310, SMD 7311) of the MA\_diff table(s).

#### Zinc Genes Identified By Correlation To Genes That Cause Physiological Consequences

Additionally, the differential expression data and the phenotypic observations can be merged to identify pathways or networks of Zinc genes. A group in the MA\_clust is considered a Zinc pathway or network if the group comprises a cDNA ID that also appears in Knock-in or Knock-out tables that causes one or more of the phenotypes described in section above.

Zinc Genes Identified By Amino Acid Sequence Similarity

Zinc genes from other plant species typically encode polypeptides that share amino acid similarity to the sequences encoded by corn and Arabidopsis Zinc genes. Groups of Zinc genes are identified in the Protein Group table. In this table, any protein group that comprises a peptide ID that corresponds to a cDNA ID member of a Zinc pathway or network is a group of proteins that also exhibits Zinc functions/utilities.

III.E.17.a. Use Of Zn Transport, Tolerance And Nutrition-Related Genes To Modulate Phenotypes

Changes in zinc concentration in the surrounding environment or in contact with a plant results in modulation of many genes and gene products. Examples of such zinc responsive genes and gene products are shown in the Reference, Sequence tables, Protein Group, Protein Group Matrix, MA\_diff, and MA\_clust tables. These genes and/or products are responsible for effects on traits such as plant vigor and seed yield.

While zinc responsive polynucleotides and gene products can act alone, combinations of these polynucleotides also affect growth and development. Useful combinations include different zinc responsive polynucleotides and/or gene products that have similar transcription profiles or similar biological activities, and members of the same or similar biochemical pathways. In addition, the combination of a zinc responsive polynucleotide and/or gene product with another environmentally responsive polynucleotide is also useful because of the interactions that exist between hormone-regulated pathways, stress pathways, nutritional pathways and development. Here, in addition to polynucleotides having similar transcription profiles and/or biological activities, useful combinations include polynucleotides that may have different transcription profiles but which participate in a common pathway.

Such zinc responsive genes and gene products can function to either increase or dampen the above phenotypes or activities either

- in response to changes in zinc concentration or
- in the absence of zinc fluctuations.

Zn transport, tolerance and nutrition-related genes and gene products can be used to alter or modulate one or more phenotypes including Zn uptake; transport of Zn or other heavy metals into

roots; epidermal/cortical uptake; Xylem loading; Zn compartmentation; Xylem unloading; Phloem loading; efflux from cells to apoplast; sequestration in vacuoles/subcellular compartments; Zn tolerance; chelation of Zn; transport of Zn; metabolic and transcriptional control; activity of Zn binding enzymes; and activity of Zn binding transcription factors.

To improve any of the phenotype(s) above, activities of one or more of the Zn transport, tolerance and nutrition-related genes or gene products can be modulated and the plants can be tested by screening for the desired trait. Specifically, the gene, mRNA levels, or protein levels can be altered in a plant utilizing the procedures described herein and the phenotypes can be assayed, for example, in accordance to Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV. J Exp Bot. 2000 Jan;51(342):71-9; Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D. Proc Natl Acad Sci U S A. 1998 Jun 9;95(12):7220-4; Crowder MW, Maiti MK, Banovic L, Makaroff CA. FEBS Lett. 1997 Dec 1;418(3):351-4; Hart JJ, Norvell WA, Welch RM, Sullivan LA, Kochian LV. Plant Physiol. 1998 Sep;118(1):219-26.

III.E.17.b. Use Of Zn Transport, Tolerance And Nutrition-Related Genes To Modulate Biochemical Activities

Alternatively, the activities of one or more of the zinc responsive genes can be modulated to change biochemical or metabolic activities and/or pathways such as those noted below. Such biological activities can be measured according to the citations included in the Table below:

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Zn Uptake and Assimilation	Zn Influx	Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV. J Exp Bot. 2000 Jan;51(342):71-9.
	Zn compartmentation	Hart JJ, Norvell WA, Welch RM, Sullivan LA, Kochian LV. Plant Physiol. 1998 Sep;118(1):219-26.

PROCESS	BIOCHEMICAL OR METABOLIC ACTIVITIES AND/OR PATHWAYS	CITATIONS INCLUDING ASSAYS
Zn binding by metabolic enzymes		Crowder MW, Maiti MK, Banovic L, Makaroff CA. FEBS Lett. 1997 Dec 1;418(3):351-4; Kenzior AL, Folk WR. FEBS Lett. 1998 Dec 4;440(3):425-9.
Zn binding by transcription factors		Newton AL, Sharpe BK, Kwan A, Mackay JP, Crossley M. J Biol Chem. 2000 May 19;275(20):15128-34; Lopez Ribera I, Ruiz-Avila L, Puigdomenech P. Biochem Biophys Res Commun. 1997 Jul 18;236(2):510-6; de Pater S, Greco V, Pham K, Memelink J, Kijne J. Nucleic Acids Res. 1996 Dec 1;24(23):4624-31.
Synthesis of proteins to chelate Zn and other metals		Schafer HJ, Greiner S, Rausch T, Haag-Kerwer A. FEBS Lett. 1997 Mar 10;404(2-3):216-20.  Rauser WE. Cell Biochem Biophys. 1999;31(1):19-48.
Synthesis of metabolites to chelate Zn and other metals		Rauser WE. Cell Biochem Biophys. 1999;31(1):19-48.

Other biological activities that can be modulated by Zn transport, tolerance and nutrition-related genes and their products are listed in the Reference tables. Assays for detecting such biological activities are described in the Protein Domain table.

Zn transport, tolerance and nutrition-related genes are differentially transcribed in response to low Zn concentrations. The microarray comparison consists of probes prepared from root RNA of *A. thaliana* (Columbia) seedlings hydroponically grown in complete nutrient medium (control) and Zn deficient seedlings grown in -Zn nutrient medium (experimental). The data from this experiment reveal a number of types genes and gene products. MA\_diff table reports the changes in transcript levels of various zinc responsive genes in entire seedlings at 1 and 6 hours after a plant was sprayed with a Hoagland's solution enriched with zinc as compared to seedlings sprayed with Hoagland's solution only.

The data from this time course can be used to identify a number of types of zinc responsive genes and gene products, including "early responding," "high zinc responders," "repressors of zinc deprivation pathways" and "zinc deprivation responders." Profiles of these different zinc responsive genes are shown in the Table below with examples of associated biological activities.

TRANSCRIPT LEVELS	TYPE OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY
Upregulated transcripts (level at 1 hour ≈ 6 hours) (level at 1 hour > 6 hours)	Early responders to Zinc	- Zinc Perception - Zinc Uptake - Modulation of Zinc Response Transduction Pathways - Specific Gene Transcription Initiation	- Transcription Factors - Transporters
	Zinc Deprivation Responders	- Repression of Pathways to Optimize Zinc Response Pathways	- Inhibit Transport of Zinc - Degradation

TRANSCRIPT LEVELS	TYPE OF GENE	PHYSIOLOGICAL CONSEQUENCE	EXAMPLES OF BIOCHEMICAL ACTIVITY
Level at 1 hour < 6 hours	Delayed Zinc Responders		-Zinc Metabolic Pathways
	Repressor of Zinc Deprivation Pathways	Negative Regulation of Zinc Pathways	
Down Regulated transcripts (Level at 1 hour $\geq$ 6 hours)  (Level at 6 hours > 1 hour)	Early responder repressors of Zinc utilization Pathways	Negative Regulators of Zinc Utilization Pathways	Suppressing Zinc Requiring processes
Level at 1 hour > 6 hours	Genes with discontinued expression or unTable mRNA following Zinc uptake	Changes in pathways and processes operating I cells	

#### USE OF PROMOTERS OF ZINC RESPONSIVE GENES

Promoters of Zinc responsive genes are useful for transcription of any desired polynucleotide or plant or non-plant origin. Further, any desired sequence can be transcribed in a similar temporal, tissue, or environmentally specific patterns as the Zinc responsive genes where the desired sequence is operably linked to a promoter of a Zinc responsive gene. The protein product of such a polynucleotide is usually synthesized in the same cells, in response to the same stimuli as the protein product of the gene from which the promoter was derived. Such promoters are also useful to produce antisense mRNAs to down-regulate the product of proteins, or to produce sense mRNAs to down-regulate mRNAs via sense suppression.

#### IV. UTILITIES OF PARTICULAR INTEREST

Genes capable of modulating the phenotypes in the following table are useful to produce the associated utilities in the table. Such genes can be identified by their cDNA ID number in the Knock-in and Knock-out Tables. That is, those genes noted in those Tables to have a phenotype as listed in the following column entitled "Phenotype Modulated by a Gene" are useful for the purpose identified in the corresponding position in the column entitled "Utilities".

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>
Leaf shape	
Cordate	decrease wind opacity,
Cup-shaped	decrease lodging (plant fall over),
Curled	increase biomass by making larger or different shaped leaves,
Laceolate	improve the efficiency of mechanical harvesting,
Lobed	decrease transpiration for better drought tolerance,
Oval	changing leaf shape to collect and absorb water,
Ovate	modulation of canopy structure and shading for altered irradiance close to the ground,
Serrate	enhanced uptake of pesticides (herbicides, fungicides, etc),
Trident	creation of ornamental leaf shapes,
Undulate	increase resistance to pathogens by decreasing amount of water that collects on leaves,
Vertically Oblong	change proportion of cell types in the leaves for enhanced photosynthesis, decreased transpiration, and enhanced
Other Shapes	accumulation of desirable compounds including secondary metabolites in specialized cells, decrease insect feeding,
Long petioles	decrease wind opacity,
Short petioles	decrease lodging (plant fall over),

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>
	increase biomass by better positioning of the leaf blade, decrease insect feeding, decrease transpiration for better drought tolerance, position leaves most effectively for photosynthetic efficiency
Fused	ornamental applications to make distinctive plants,
Reduced fertility	
Short siliques	increase or decrease the number of seeds in a fruit, increasing fruit size, modulating fruit shape to better fit harvesting or packaging requirements, useful for controlling dehiscence and seed scatter
Reduced fertility Sterility	useful in hybrid breeding programs, increasing fruit size, production of seedless fruit, useful as targets for gametocides, modulating fruit shape to better fit harvesting or packaging requirements, useful for controlling dehiscence and seed scatter
Flower size	useful for edible flowers useful for flower derived products such as fragrances useful for modulating seed size and number in combination with seed-specific genes value in the ornamental industry
Stature	
Large	increasing or decreasing plant biomass, optimizing plant stature to increase yield under various diverse environmental conditions, e.g., when water or nutrients
Small	

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>	
Dwarfs	are limiting, decreasing lodging, increasing fruit number and size, controlling shading and canopy effects	
Meristems	Change plant architecture, increase or decrease number of leaves as well as change the types of leaves to increase biomass, improve photosynthetic efficiency, create new varieties of ornamental plants with enhanced leaf design, preventing flowering to optimize vegetative growth, control of apical dominance, increase or decrease flowering time to fit season, water or fertilizer schedules, change arrangement of leaves on the stem (phyllotaxy) to optimize plant density, decrease insect feeding, or decrease pathogen infection, increase number of trichome/glandular trichome producing leaves targets for herbicides, generate ectopic meristems and ectopic growth of vegetative and floral tissues and seeds and fruits	
Stem	Strong  Weak	modify lignin content/composition for creation of harder woods or reduce difficulty/costs in pulping for paper production or increase digestibility of forage crops, decrease lodging, modify cell wall polysaccharides in stems and fruits for improved texture and nutrition. increase biomass

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>
Late/Early Bolting	Break the need for long vernalization of vernalization-dependent crops, e.g., winter wheat, thereby increasing yield decrease or increase generation time increase biomass
Lethals	Embryo-lethal produce seedless fruit, use as herbicide targets
	Embryo-defective produce seedless fruit, use as herbicide targets
	Seedling use as herbicide targets, useful for metabolic engineering,
	Pigment-lethals use as herbicide targets, increase photosynthetic efficiency
Pigment	Dark Green Increase nutritional value, enhanced photosynthesis and carbon dioxide combustion and therefore increase plant vigor and biomass, enhanced photosynthetic efficiency and therefore increase plant vigor and biomass, prolong vegetative development, enhanced protection against pathogens,
YGV1	Useful as targets for herbicides, increase photosynthetic efficiency and therefore increase plant vigor and biomass,
YGV2	Useful as targets for herbicides, control of change from embryonic to adult organs, increase metabolic efficiency, increase photosynthetic efficiency and therefore increased plant vigor and biomass,

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>	
YGV3	Useful as targets for herbicides, nitrogen sensing/uptake/usage, increase metabolic efficiency and therefore increased plant vigor and biomass,	
Interveinal chlorosis	to increase photosynthetic efficiency and therefore increase plant vigor and biomass to increase or decrease nitrogen transport and therefore increase plant vigor and biomass use as herbicide targets increase metabolic efficiency,	
Roots	Short (primary root)  Thick (primary root)  Branching (primary root)  Long (lateral roots)	to access water from rainfall, to access rhizobia spray application, for anaerobic soils, useful to facilitate harvest of root crops,  useful for increasing biomass of root crops, for preventing plants dislodging during picking and harvesting, as root grafts, for animal feeds  modulation allows better access to water, minerals, fertilizers, rhizobia prevent soil erosion,s increasing root biomass decrease root lodging,  modulation allows improved access to water, nutrients, fertilizer, rhizobia, prevent soil erosion increase root biomass decrease root lodging modulation allows control on the depth of root growth in soil to access water and nutrients modulation allows hormonal control of root growth and development (size)

<u>Phenotype</u> <u>Modulated by a</u> <u>Gene</u>	<u>Utilities</u>
Agravitropic	modulation allows control on the depth of root growth in soil
Curling (primary root)	modulation allows hormonal control of root growth and development (size) useful in anaerobic soils in allowing roots to stay close to surface harvesting of root crops
Poor germination	
Trichome	Reduced Number Glabrous
	Increased Number
Wax mutants	Genes useful for decreasing transpiration, increased production of glandular trichomes for oil or other secreted chemicals of value, use as deterrent for insect herbivory and oviposition modulation will increase resistance to UV light,
Cotyledons	decrease insect herbivory and oviposition, composition changes for the cosmetics industry, decrease transpiration, provide pathogen resistance, UV protection, modulation of leaf runoff properties and improved access for herbicides and fertilizers
Seeds	modulation of seeds structure in legumes, increase nutritional value, improve seedling competition under field conditions,
	Transparent testa
	Light
	Dark
	genes useful for metabolic engineering anthocyanin and flavonoid pathways improved nutritional content decrease petal abscission

<u>Phenotype Modulated by a Gene</u>		<u>Utilities</u>
Flowers	Other	decrease pod shattering
Hypocotyls	Long	to improve germination rates to improve plant survivability
	Short	to improve germination rates to improve plant survivability

## V. ENHANCED FOODS

Animals require external supplies of amino acids that they cannot synthesize themselves. Also, some amino acids are required in larger quantities. The nutritional values of plants for animals and humans can thus be modified by regulating the amounts of the constituent amino acids that occur as free amino acids or in proteins. For instance, higher levels of lysine and/or methionine would enhance the nutritional value of corn seed. Applicants herein provide several methods for modulating the amino acid content:

- (1) expressing a naturally occurring protein that has a high percentage of the desired amino acid(s);
- (2) expressing a modified or synthetic coding sequence that has an enhanced percentage of the desired amino acids; or
- (3) expressing the protein(s) that are capable of synthesizing more of the desired amino acids.

A specific example is expressing proteins with enhanced, for example, methionine content, preferentially in a corn or cereal seed used for animal nutrition or in the parts of plants used for nutritional purposes.

A protein is considered to have a high percentage of an amino acid if the amount of the desired amino acid is at least 1% of the total number of residues in a protein; more preferably 2% or greater. Amino acids of particular interest are tryptophan, lysine, methionine, phenylalanine, threonine leucine, valine, and isoleucine. Examples of naturally occurring proteins with a high

percentage of any one of the amino acid of particular interest are listed in the Enhanced Amino Acid Table.

The sequence(s) encoding the selected protein(s) are operably linked to a promoter and other regulatory sequences and transformed into a plant as described below. The promoter is chosen optimally for promoting the desired level of expression of the protein in the selected organ e.g. a promoter highly functional in seeds. Modifications may be made to the sequence encoding the protein to ensure protein transport into, for example, organelles or storage bodies or its accumulation in the organ. Such modifications may include addition of signal sequences at or near the N terminus and amino acid residues to modify protein stability or appropriate glycosylation. Other modifications may be made to the transcribed nucleic acid sequence to enhance the stability or translatability of the mRNA, in order to ensure accumulation of more of the desired protein. Suitable versions of the gene construct and transgenic plants are selected on the basis of, for example, the improved amino acid content and nutritional value measured by standard biochemical tests and animal feeding trials.

#### **VI. USE OF NOVEL GENES TO FACILITATE EXPLOITATION OF PLANTS AS FACTORIES FOR THE SYNTHESIS OF VALUBLE MOLECULES**

Plants and their constituent cells, tissues, and organs are factories that manufacture small organic molecules such as sugars, amino acids, fatty acids, vitamins, etc., as well as macromolecules such as proteins, nucleic acids, oils/fats and carbohydrates. Plants have long been a source of pharmaceutically beneficial chemicals; particularly, the secondary metabolites and hormone-related molecules synthesized by plants. Plants can also be used as factories to produce carbohydrates or lipids that comprises a carbon backbone useful as precursors of plastics, fiber, fuel, paper, pulp, rubber, solvents, lubricants, construction materials, detergents, and other cleaning materials. Plants can also generate other compounds that are of economic value, such as dyes, flavors, and fragrances. Both the intermediates as well as the end-products of plant bio-synthetic pathways have been found useful.

With the polynucleotides and polypeptides of the instant invention, modification of both in-vitro and in-vivo synthesis of such products is possible. One method of increasing the amount of either the intermediates or the end-products synthesized in a cell is to increase the expression

of one or more proteins in the synthesis pathway as discussed below. Another method of increasing production of an intermediate is to inhibit expression of protein(s) that synthesize the end-product from the intermediate. Levels of end-products and intermediates can also be modified by changing the levels of enzymes that specifically change or degrade them. The kinds of molecules made can be also be modified by changing the genes encoding specific enzymes performing reactions at specific steps of the biosynthetic pathway. These genes can be from the same or a different organism. The molecular structures in the biosynthetic pathways can thus be modified or diverted into different branches of a pathway to make novel end-products.

Novel genes comprising selected promoters and sequences encoding enzymes are transformed into the selected plant to modify the levels, composition and/or structure of, without limitation:

- Terpenoids
- Alkaloids
- Hormones, including brassinosteroids
- Flavonoids
- Steroids
- Vitamins such as
  - Retinol
  - Riboflavin
  - Thiamine
- Caffeine
- Morphine and other alkaloids
- Peptides and amino acid synthesis
- Antioxidants
- Starches and lipids
- Fatty acids
- Fructose, mannose and other sugars
- Glycerolipid
- Citric acid
- Lignin

- Flavors
- Fragrances
- Essential oils
- Colors or dyes
- Gum
- Gels
- Waxes

The modifications are made by designing one or more novel genes per application comprising promoters, to ensure production of the enzyme(s) in the relevant cells, in the right amount, and polynucleotides encoding the relevant enzyme. The promoters and polynucleotides are the subject of this application. The novel genes are transformed into the relevant species using standard procedures. Their effects are measured by standard assays for the specific chemical/biochemical products.

These polynucleotides and proteins of the invention that participate in the relevant pathways and are useful for changing production of the above chemicals and biochemicals are identified in the Reference tables by their enzyme function. More specifically, proteins of the invention that have the enzymatic activity of one of the entries in the following table entitled "Enzymes Effecting Modulation of Biological Pathways" are of interest to modulate the corresponding pathways to produce precursors or final products noted above that are of industrial use. Biological activities of particular interest are listed below.

Other polynucleotides and proteins that regulate where, when and to what extent a pathway is active in a plant are extremely useful for modulating the synthesis and accumulation of valuable chemicals. These elements including transcription factors, proteins involved in signal transduction and other proteins in the control of gene expression are described elsewhere in this application.

Pathway Name	Enzyme Description	Comments
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Alkaloid biosynthesis I	Morphine 6-dehydrogenase	Also acts on other alkaloids, including codeine, normorphine and ethylmorphine, but only very slowly on 7,8-saturated derivatives such as dihydromorphine and dihydrocodeine In the reverse direction, also reduces naloxone to the 6-alpha-hydroxy analog Activated by 2-mercaptoethanol
	Codeinone reductase (NADPH)	Stereospecifically catalyses the reversible reduction of codeinone to codeine, which is a direct precursor of morphine in the opium poppy plant, <i>Papaver somniferum</i>
	Salutaridine reductase (NADPH)	Stereospecifically catalyses the reversible reduction of salutaridine to salutaridinol, which is a direct precursor of morphinan alkaloids in the poppy plant, <i>Papaver somniferum</i>
	(S)-stylopine synthase	Catalyses an oxidative reaction that does not incorporate oxygen into the product Forms the second methylenedioxy bridge of the protoberberine alkaloid stylopine from oxidative ring closure of adjacent phenolic and methoxy groups of cheilanthifoline
	(S)-cheilanthifoline synthase	Catalyses an oxidative reaction that does not incorporate oxygen into the product Forms the methylenedioxy bridge of the protoberberine alkaloid cheilanthifoline from oxidative ring closure of adjacent phenolic and methoxy groups of scoulerine
	Salutaridine synthase	Forms the morphinan alkaloid salutaridine by intramolecular phenol oxidation of reticuline without the incorporation of oxygen into the product
	(S)-canadine synthase	Catalyses an oxidative reaction that does not incorporate oxygen into the product Oxidation of the methoxyphenol group of the alkaloid tetrahydrocolumbamine results in the formation of the methylenedioxy bridge of canadine
	Protopine 6-monooxygenase	Involved in benzophenanthridine alkaloid synthesis in higher plants
	Dihydrosanguinarine 10-monooxygenase	Involved in benzophenanthridine alkaloid synthesis in higher plants
	Monophenol monooxygenase	A group of copper proteins that also catalyse the reaction of EC 1.10.3.1, if only 1,2-benzenediols are available as substrate

	L-amino acid oxidase	
	1,2-dehydroreticulinium reductase (NADPH)	Stereospecifically reduces the 1,2-dehydroreticulinium ion to (R)-reticuline, which is a direct precursor of morphinan alkaloids in the poppy plant, <i>papaver somniferum</i> The enzyme does not catalyse the reverse reaction to any significant extent under physiological conditions
	Dihydrobenzophenanthridine oxidase	Also catalyzes: dihydrochelirubine + O(2) = chelirubine + H(2)O(2) Also catalyzes: dihydromacarpine + O(2) = macarpine + H(2)O(2) Found in higher plants Produces oxidized forms of the benzophenanthridine alkaloids
	Reticuline oxidase	The product of the reaction, (S)-scoulerine, is a precursor of protopine, protoberberine and benzophenanthridine alkaloid biosynthesis in plants Acts on (S)-reticuline and related compounds, converting the N- methyl group into the methylene bridge ('berberine bridge[PRIME]) of (S)-tetrahydroprotoberberines
	3[PRIME]-hydroxy-N-methyl-(S)-coclaurine 4[PRIME]-O-methyltransferase	Involved in isoquinoline alkaloid metabolism in plants Has also been shown to catalyse the methylation of (R,S)- laudanosoline, (S)-3[PRIME]-hydroxycoclaurine and (R,S)-7-O-methylnorlaudanosoline
	(S)-scoulerine 9-O-methyltransferase	The product of this reaction is a precursor for protoberberine alkaloids in plants
	Columbamine O-methyltransferase	The product of this reaction is a protoberberine alkaloid that is widely distributed in the plant kingdom Distinct in specificity from EC 2.1.1.88
	10-hydroxydihydro-sanguinarine 10-O-methyltransferase	Part of the pathway for synthesis of benzophenanthridine alkaloids in plants
	12-hydroxydihydrochelirubine 12-O-methyltransferase	Part of the pathway for synthesis of benzophenanthridine alkaloid macarpine in plants

	(R,S)-norcoclaurine 6-O-methyltransferase	Norcoclaurine is 6,7-dihydroxy-1-[(4-hydroxyphenyl)methyl]-1,2,3,4-tetrahydroisoquinoline The enzyme will also catalyse the 6-O-methylation of (R,S)-norlaudanosoline to form 6-O-methyl-norlaudanosoline, but this alkaloid has not been found to occur in plants
	Salutaridinol 7-O-acetyltransferase	At higher pH values the product, 7-O-acetylsalutaridinol, spontaneously closes the 4->5 oxide bridge by allylic elimination to form the morphine precursor thebaine From the opium poppy plant, <i>Papaver somniferum</i>
	Aspartate aminotransferase	Also acts on L-tyrosine, L-phenylalanine and L-tryptophan. This activity can be formed from EC 2.6.1.57 by controlled proteolysis
	Tyrosine aminotransferase	L-phenylalanine can act instead of L-tyrosine The mitochondrial enzyme may be identical with EC 2.6.1.1 The three isoenzymic forms are interconverted by EC 3.4.22.4
	Aromatic amino acid transferase	L-methionine can also act as donor, more slowly Oxaloacetate can act as acceptor Controlled proteolysis converts the enzyme to EC 2.6.1.1
	Tyrosine decarboxylase	The bacterial enzyme also acts on 3-hydroxytyrosine and, more slowly, on 3-hydroxyphenylalanine
	Aromatic-L-amino-acid decarboxylase	Also acts on L-tryptophan, 5-hydroxy-L-tryptophan and dihydroxy- L-phenylalanine (DOPA)
Alkaloid biosynthesis II	Tropine dehydrogenase	Oxidizes other tropan-3-alpha-ols, but not the corresponding beta- derivatives
	Tropinone reductase	
	Hyoscyamine (6S)-dioxygenase	
	6-beta-hydroxyhyoscyamine epoxidase	
	Amine oxidase (copper-containing)	A group of enzymes including those oxidizing primary amines, diamines and histamine One form of EC 1.3.1.15 from rat kidney also catalyses this reaction
	Putrescine N-	

	methyltransferase	
	Ornithine decarboxylase	
	Oxaryl-CoA decarboxylase	
	Phenylalanine ammonia-lyase	May also act on L-tyrosine
Androgen and estrogen metabolism	3-beta-hydroxy-delta(5)-steroid dehydrogenase	Acts on 3-beta-hydroxyandrost-5-en-17-one to form androst-4-ene- 3,17-dione and on 3-beta-hydroxypregn-5-en-20-one to form progesterone
	11-beta-hydroxysteroid dehydrogenase	
	Estradiol 17-alpha-dehydrogenase	
	3-alpha-hydroxy-5-beta-androstan-17-one 3-alpha-dehydrogenase	
	3-alpha (17-beta)-hydroxysteroid dehydrogenase (NAD+)	Also acts on other 17-beta-hydroxysteroids, on the 3-alpha- hydroxy group of pregnanes and bile acids, and on benzene dihydriodiol Different from EC 1.1.1.50 or EC 1.1.1.213
	3-alpha-hydroxysteroid dehydrogenase (B-specific)	Acts on other 3-alpha-hydroxysteroids and on 9-, 11- and 15- hydroxyprostaglandin B-specific with respect to NAD(+) or NADP(+) (cf. EC 1.1.1.213)
	3(or 17)beta-hydroxysteroid dehydrogenase	Also acts on other 3-beta- or 17-beta-hydroxysteroids (cf EC 1.1.1.209)
	Estradiol 17 beta-dehydrogenase	Also acts on (S)-20-hydroxypregn-4-en-3-one and related compounds, oxidizing the (S)-20-group B-specific with respect to NAD(P)(+)
	Testosterone 17-beta-dehydrogenase	
	Testosterone 17-beta-dehydrogenase (NADP+)	Also oxidizes 3-hydroxyhexobarbital to 3-oxohexobarbital
	Steroid 11-beta-monooxygenase	Also hydroxylates steroids at the 18-position, and converts 18-hydroxycorticosterone into aldosterone
	Estradiol 6-beta-monooxygenase	

	Androst-4-ene-3,17-dione monooxygenase	Has a wide specificity A single enzyme from <i>Cylindrocarpon radicicola</i> (EC 1.14.13.54) catalyses both this reaction and that catalysed by EC 1.14.99.4
	3-oxo-5-alpha-steroid 4-dehydrogenase	
	3-oxo-5-beta-steroid 4-dehydrogenase	
	UDP-glucuronosyltransferase	Family of enzymes accepting a wide range of substrates, including phenols, alcohols, amines and fatty acids Some of the activities catalysed were previously listed separately as EC 2.4.1.42, EC 2.4.1.59, EC 2.4.1.61, EC 2.4.1.76, EC 2.4.1.77, EC 2.4.1.84, EC 2.4.1.107 and EC 2.4.1.108 A temporary nomenclature for the various forms whose delineation is in a state of flux
	Steroid sulfotransferase	Broad specificity resembling EC 2.8.2.2, but also acts on estrone
	Alcohol sulfotransferase	Primary and secondary alcohols, including aliphatic alcohols, ascorbate, chloramphenicol, ephedrine and hydroxysteroids, but not phenolic steroids, can act as acceptors (cf. EC 2.8.2.15)
	Estrone sulfotransferase	
	Arylsulfatase	A group of enzymes with rather similar specificities
	Steryl-sulfatase	Also acts on some related steryl sulfates
	17-alpha-hydroxyprogesterone aldolase	
	Steroid delta-isomerase	
C21-Steroid hormone metabolism	3-beta-hydroxy-delta(5)-steroid dehydrogenase	Acts on 3-beta-hydroxyandrost-5-en-17-one to form androst-4-ene-3,17-dione and on 3-beta-hydroxypregn-5-en-20-one to form progesterone
	11-beta-hydroxysteroid dehydrogenase	

	20-alpha-hydroxysteroid dehydrogenase	A-specific with respect to NAD(P)(+)
	3-alpha-hydroxysteroid dehydrogenase (B-specific)	Acts on other 3-alpha-hydroxysteroids and on 9-, 11- and 15- hydroxyprostaglandin B-specific with respect to NAD(+) or NADP(+) (cf. EC 1.1.1.213)
	3-alpha(or 20-beta)-hydroxysteroid dehydrogenase	The 3-alpha-hydroxyl group or 20-beta-hydroxyl group of pregnane and androstane steroids can act as donors
	Steroid 11-beta-monooxygenase	Also hydroxylates steroids at the 18-position, and converts 18-hydroxycorticosterone into aldosterone
	Corticosterone 18-monooxygenase	
	Cholesterol monooxygenase (side-chain cleaving)	The reaction proceeds in three stages, with hydroxylation at C-20 and C-22 preceding scission of the side-chain at C-20
	Steroid 21-monooxygenase	
	Progesterone 11-alpha-monooxygenase	
	Steroid 17-alpha-monooxygenase	
	Cholestenone 5-beta-reductase	
	Cortisone beta-reductase	
	Progesterone 5-alpha-reductase	Testosterone and 20-alpha-hydroxy-4-pregnen-3-one can act in place of progesterone
	3-oxo-5-beta-steroid 4-dehydrogenase	
	Steroid delta-isomerase	
Flavonoids, stilbene and lignin biosynthesis	Coniferyl-alcohol dehydrogenase	Specific for coniferyl alcohol; does not act on cinnamyl alcohol, 4-coumaryl alcohol or sinapyl alcohol

	Cinnamyl-alcohol dehydrogenase	Acts on coniferyl alcohol, sinapyl alcohol, 4-coumaryl alcohol and cinnamyl alcohol (cf. EC 1.1.1.194)
	Dihydrokaempferol 4-reductase	Also acts, in the reverse direction, on (+)-dihydroquercetin and (+)-dihydromyricetin Each dihydroflavonol is reduced to the corresponding cis-flavon-3,4-diol NAD(+) can act instead of NADP(+), more slowly Involved in the biosynthesis of anthocyanidins in plants
	Flavonone 4-reductase	Involved in the biosynthesis of 3-deoxyanthocyanidins from flavonones such as naringenin or eriodictyol
	Peroxidase	
	Caffeate 3,4-dioxygenase	
	Naringenin 3-dioxygenase	
	Trans-cinnamate 4-monooxygenase	Also acts on NADH, more slowly
	Trans-cinnamate 2-monooxygenase	
	Flavonoid 3[PRIME]-monooxygenase	Acts on a number of flavonoids, including naringenin and dihydrokaempferol Does not act on 4-coumarate or 4-coumaroyl-CoA
	Monophenol monooxygenase	A group of copper proteins that also catalyse the reaction of EC 1.10.3.1, if only 1,2-benzenediols are available as substrate
	Cinnamoyl-CoA reductase	Also acts on a number of substituted cinnamoyl esters of coenzyme A
	Caffeoyl-CoA O-methyltransferase	
	Luteolin O-methyltransferase	Also acts on luteolin-7-O-beta-D-glucoside
	Caffeate O-methyltransferase	3,4-dihydroxybenzaldehyde and catechol can act as acceptor, more slowly
	Apigenin 4[PRIME]-O-methyltransferase	Converts apigenin into acacetin Naringenin (5,7,4[PRIME]-trihydroxyflavonone) can also

		act as acceptor, more slowly
	Quercetin 3-O-methyltransferase	Specific for quercetin. Related enzymes bring about the 3-O- methylation of other flavonols, such as galangin and kaempferol
	Isoflavone-7-O-beta-glucoside 6[PRIME][PRIME]-O-malonyltransferase	The 6-position of the glucose residue of formononetin can also act as acceptor Some other 7-O-glucosides of isoflavones, flavones and flavonols can also act, more slowly
	Pinosylvin synthase	Not identical with EC 2.3.1.74 or EC 2.3.1.95
	Naringenin-chalcone synthase	In the presence of NADH and a reductase, 6[PRIME]-deoxychalcone is produced
	Trihydroxystilbene synthase	Not identical with EC 2.3.1.74 or EC 2.3.1.146
	Quinate O-hydroxycinnamoyltransferase	Caffeoyl-CoA and 4-coumaroyl-CoA can also act as donors, more slowly Involved in the biosynthesis of chlorogenic acid in sweet potato and, with EC 2.3.1.98 in the formation of caffeoyl-CoA in tomato
	Coniferyl-alcohol glucosyltransferase	Sinapyl alcohol can also act as acceptor
	2-coumarate O-beta-glucosyltransferase	Coumarinate (cis-2-hydroxycinnamate) does not act as acceptor
	Scopoletin glucosyltransferase	
	Flavonol-3-O-glucoside L-rhamnosyltransferase	Converts flavonol 3-O-glucosides to 3-O-rutinosides Also acts, more slowly, on rutin, quercetin 3-O-galactoside and flavonol O3-rhamnosides
	Flavone 7-O-beta-glucosyltransferase	A number of flavones, flavonones and flavonols can function as acceptors Different from EC 2.4.1.91
	Flavonol 3-O-glucosyltransferase	Acts on a variety of flavonols, including quercetin and quercetin 7-O-glucoside Different from EC 2.4.1.81
	Flavone apiosyltransferase	7-O-beta-D-glucosides of a number of flavonoids and of 4-substituted phenols can act as acceptors
	Coniferin beta-glucosidase	Also hydrolyzes syringin, 4-cinnamyl alcohol beta-glucoside, and, more slowly, some other aryl beta-glycosides A plant cell-wall enzyme involved in the biosynthesis of lignin

	Beta-glucosidase	Wide specificity for beta-D-glucosides. Some examples also hydrolyse one or more of the following: beta-D-galactosides, alpha-L-arabinosides, beta-D-xylosides, and beta-D-fucosides
	Chalcone isomerase	
	4-coumarate--CoA ligase	

Pathway Name	Enzyme Description	Enzyme Comments
Ascorbate and aldarate metabolism	D-threo-aldoose 1-dehydrogenase	Acts on L-fucose, D-arabinose and L-xylose The animal enzyme was also shown to act on L-arabinose, and the enzyme from <i>Pseudomonas caryophylli</i> on L-glucose
	L-threonate 3-dehydrogenase	
	Glucuronate reductase	Also reduces D-galacturonate May be identical with EC 1.1.1.2
	Glucuronolactone reductase	
	L-arabinose 1-dehydrogenase	
	L-galactonolactone oxidase	Acts on the 1,4-lactones of L-galactonic, D-altronic, L-fuconic, D-arabinic and D-threonic acids Not identical with EC 1.1.3.8 (cf. EC 1.3.2.3)
	L-gulonolactone oxidase	The product spontaneously isomerizes to L-ascorbate
	L-ascorbate oxidase	
	L-ascorbate peroxidase	
	Ascorbate 2,3-dioxygenase	
	2,5-dioxovalerate dehydrogenase	
	Aldehyde dehydrogenase (NAD+)	Wide specificity, including oxidation of D-glucuronolactone to D-glucarate
	Galactonolactone dehydrogenase	Cf. EC 1.1.3.24
	Monodehydroascorbate reductase (NADH)	
	Glutathione dehydrogenase (ascorbate)	
	L-arabinonolactonase	
	Gluconolactonase	Acts on a wide range of hexono-1,5-lactones
	Uronolactonase	
	1,4-lactonase	Specific for 1,4-lactones with 4-8 carbon atoms Does not hydrolyse simple

		aliphatic esters, acetylcholine, sugar lactones or substituted aliphatic lactones, e.g. 3-hydroxy-4- butyrolactone
	2-dehydro-3-deoxyglucarate aldolase	
	L-arabinonate dehydratase	
	Glucarate dehydratase	
	5-dehydro-4-deoxyglucarate dehydratase	
	Galactarate dehydratase	
	2-dehydro-3-deoxy-L-arabinonate dehydratase	
Carbon fixation	Malate dehydrogenase	Also oxidizes some other 2-hydroxydicarboxylic acids
	Malate dehydrogenase (decarboxylating)	Does not decarboxylates added oxaloacetate
	Malate dehydrogenase (oxaloacetate decarboxylating) (NADP+)	Also decarboxylates added oxaloacetate
	Malate dehydrogenase (NADP+)	Activated by light
	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating)	
	Transketolase	Wide specificity for both reactants, e.g. converts hydroxypyruvate and R-CHO into CO(2) and R-CHOH-CO-CH(2)OH Transketolase from Alcaligenes faecalis shows high activity with D-erythrose as acceptor
	Aspartate aminotransferase	Also acts on L-tyrosine, L-phenylalanine and L-tryptophan. This activity can be formed from EC 2.6.1.57 by controlled proteolysis
	Alanine aminotransferase	2-aminobutanoate acts slowly instead of alanine
	Sedoheptulokinase	
	Phosphoribulokinase	

	Pyruvate kinase	UTP, GTP, CTP, ITP and dATP can also act as donors Also phosphorylates hydroxylamine and fluoride in the presence of CO(2)
	Phosphoglycerate kinase	
	Pyruvate,phosphate dikinase	
	Fructose-bisphosphatase	The animal enzyme also acts on sedoheptulose 1,7-bisphosphate
	Sedoheptulose-bisphosphatase	
	Phosphoenolpyruvate carboxylase	
	Ribulose-bisphosphate carboxylase	Will utilize O(2) instead of CO(2), forming 3-phospho-D-glycerate and 2-phosphoglycolate
	Phosphoenolpyruvate carboxykinase (ATP)	
	Fructose-bisphosphate aldolase	Also acts on (3S,4R)-ketose 1-phosphates The yeast and bacterial enzymes are zinc proteins The enzymes increase electron-attraction by the carbonyl group, some (Class I) forming a protonated imine with it, others (Class II), mainly of microbial origin, polarizing it with a metal ion, e.g zinc
	Phosphoketolase	
	Ribulose-phosphate 3-epimerase	Also converts D-erythrose 4-phosphate into D-erythrulose 4-phosphate and D-threose 4-phosphate
	Triosephosphate isomerase	
	Ribose 5-phosphate epimerase	Also acts on D-ribose 5-diphosphate and D-ribose 5-triphosphate
Phenylalanine metabolism	(R)-4-hydroxyphenyllactate dehydrogenase	Also acts, more slowly, on (R)-3-phenyllactate, (R)-3-(indole-3-yl)lactate and (R)-lactate
	Hydroxyphenylpyruvate reductase	Also acts on 3-(3,4-dihydroxyphenyl)lactate Involved with EC 2.3.1.140 in the biosynthesis of rosmarinic acid
	Aryl-alcohol dehydrogenase	A group of enzymes with broad specificity towards primary alcohols with

		an aromatic or cyclohex-1-ene ring, but with low or no activity towards short-chain aliphatic alcohols
	Peroxidase	
	Catechol 1,2-dioxygenase	Involved in the metabolism of nitroaromatic compounds by a strain of <i>Pseudomonas putida</i>
	2,3-dihydroxybenzoate 3,4-dioxygenase	
	3-carboxyethylcatechol 2,3-dioxygenase	
	Catechol 2,3-dioxygenase	The enzyme from <i>Alcaligenes</i> sp. strain O-1 has also been shown to catalyse the reaction: 3-Sulfocatechol + O(2) + H(2)O = 2-hydroxymuconate + bisulfite. It has been referred to as 3-sulfocatechol 2,3-dioxygenase. Further work will be necessary to show whether or not this is a distinct enzyme
	4-hydroxyphenylpyruvate dioxygenase	
	Protocatechuate 3,4-dioxygenase	
	Hydroxyquinol 1,2-dioxygenase	The product isomerizes to 2-maleylacetate (cis-hex-2-enedioate). Highly specific; catechol and pyrogallol are acted on at less than 1% of the rate at which benzene-1,2,4-triol is oxidized
	Protocatechuate 4,5-dioxygenase	
	Phenylalanine 2-monoxygenase	Also catalyses a reaction similar to that of EC 1.4.3.2, forming 3-phenylpyruvate, NH(3) and H(2)O(2), but more slowly
	Anthranilate 1,2-dioxygenase (deaminating, decarboxylating)	
	Benzoate 1,2-dioxygenase	A system, containing a reductase which is an iron-sulfur flavoprotein (FAD), and an iron-sulfur oxygenase
	Toluene dioxygenase	A system, containing a reductase which is an iron-sulfur flavoprotein (FAD), an iron-sulfur oxygenase, and a ferredoxin

		Some other aromatic compounds, including ethylbenzene, 4-xylene and some halogenated toluenes, are converted into the corresponding cis-dihydrodiols
	Naphthalene 1,2-dioxygenase	A system, containing a reductase which is an iron-sulfur flavoprotein (FAD), an iron-sulfur oxygenase, and ferredoxin
	Benzene 1,2-dioxygenase	A system, containing a reductase which is an iron-sulfur flavoprotein, an iron-sulfur oxygenase and ferredoxin
	Salicylate 1-monoxygenase	
	Trans-cinnamate 4-monoxygenase	Also acts on NADH, more slowly
	Benzoate 4-monoxygenase	
	4-hydroxybenzoate 3-monoxygenase	Most enzymes from Pseudomonas are highly specific for NAD(P)H (cf EC 1.14.13.33)
	3-hydroxybenzoate 4-monoxygenase	Also acts on a number of analogs of 3-hydroxybenzoate substituted in the 2, 4, 5 and 6 positions
	3-hydroxybenzoate 6-monoxygenase	Also acts on a number of analogs of 3-hydroxybenzoate substituted in the 2, 4, 5 and 6 positions NADPH can act instead of NADH, more slowly
	4-hydroxybenzoate 3-monoxygenase (NAD(P)H)	The enzyme from <i>Corynebacterium cyclohexanicum</i> is highly specific for 4-hydroxybenzoate, but uses NADH and NADPH at approximately equal rates (cf. EC 1.14.13.2). It is less specific for NADPH than EC 1.14.13.2
	Anthranilate 3-monoxygenase (deaminating)	The enzyme from <i>Aspergillus niger</i> is an iron protein; that from the yeast <i>Trichosporon cutaneum</i> is a flavoprotein (FAD)
	Melilotate 3-monoxygenase	
	Phenol 2-monoxygenase	Also active with resorcinol and O-cresol
	Mandelate 4-monoxygenase	
	3-hydroxybenzoate 2-monoxygenase	

	4-cresol dehydrogenase (hydroxylating)	Phenazine methosulfate can act as acceptor A quinone methide is probably formed as intermediate The product is oxidized further to 4-hydroxybenzoate
	Benzaldehyde dehydrogenase (NAD+)	
	Aminomuconate-semialdehyde dehydrogenase	Also acts on 2-hydroxymuconate semialdehyde
	Phenylacetaldehyde dehydrogenase	
	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase	Does not act on unsubstituted aliphatic or aromatic aldehydes or glucose NAD(+) can replace NADP(+), but with lower affinity
	Aldehyde dehydrogenase (NAD(P)+)	
	Benzaldehyde dehydrogenase (NADP+)	
	Coumarate reductase	
	Cis-1,2-dihydrobenzene-1,2-diol dehydrogenase	
	Cis-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase	Also acts, at half the rate, on cis-anthracene dihydriodiol and cis-phenanthrene dihydriodiol
	2-enoate reductase	Acts, in the reverse direction, on a wide range of alkyl and aryl alpha,beta-unsaturated carboxylate ions 2-butenoate was the best substrate tested
	Maleylacetate reductase	
	Phenylalanine dehydrogenase	The enzyme from <i>Bacillus badius</i> and <i>Sporosarcina ureae</i> are highly specific for L-phenylalanine, that from <i>Bacillus sphaericus</i> also acts on L-tyrosine
	L-amino acid oxidase	
	Amine oxidase (flavin-containing)	Acts on primary amines, and usually also on secondary and tertiary amines
	Amine oxidase (copper-containing)	A group of enzymes including those oxidizing primary amines, diamines and histamine One form of EC 1.3.1.15 from rat kidney also catalyses this reaction

	D-amino-acid dehydrogenase	Acts to some extent on all D-amino acids except D-aspartate and D-glutamate
	Aralkylamine dehydrogenase	Phenazine methosulfate can act as acceptor Acts on aromatic amines and, more slowly, on some long-chain aliphatic amines, but not on methylamine or ethylamine (cf EC 1.4.99.3)
	Glutamine N-phenylacetyltransferase	
	Acetyl-CoA C-acyltransferase	
	D-amino-acid N-acetyltransferase	
	Phenylalanine N-acetyltransferase	Also acts, more slowly, on L-histidine and L-alanine
	Glycine N-benzoyltransferase	Not identical with EC 2.3.1.13 or EC 2.3.1.68
	Aspartate aminotransferase	Also acts on L-tyrosine, L-phenylalanine and L-tryptophan. This activity can be formed from EC 2.6.1.57 by controlled proteolysis
	D-alanine aminotransferase	Acts on the D-isomers of leucine, aspartate, glutamate, aminobutyrate, norvaline and asparagine
	Tyrosine aminotransferase	L-phenylalanine can act instead of L-tyrosine The mitochondrial enzyme may be identical with EC 2.6.1.1 The three isoenzymic forms are interconverted by EC 3.4.22.4
	Aromatic amino acid transferase	L-methionine can also act as donor, more slowly Oxaloacetate can act as acceptor Controlled proteolysis converts the enzyme to EC 2.6.1.1
	Histidinol-phosphate aminotransferase	
	3-oxoadipate CoA-transferase	
	3-oxoadipate enol-lactonase	Acts on the product of EC 4.1.1.44
	Carboxymethylene-butenolidase	
	2-pyrone-4,6-dicarboxylate lactonase	The product isomerizes to 4-oxalomesaconate

	Hippurate hydrolase	Acts on various N-benzoylamino acids
	Amidase	
	Acylphosphatase	
	2-hydroxymuconate-semialdehyde hydrolase	
	Aromatic-L-amino-acid-decarboxylase	Also acts on L-tryptophan, 5-hydroxy-L-tryptophan and dihydroxy- L-phenylalanine (DOPA)
	Phenylpyruvate-decarboxylase	Also acts on indole-3-pyruvate
	4-carboxymuconolactone decarboxylase	
	O-pyrocatechuate-decarboxylase	
	Phenylalanine-decarboxylase	Also acts on tyrosine and other aromatic amino acids
	4-hydroxybenzoate-decarboxylase	
	Protocatechuate-decarboxylase	
	Benzoylformate-decarboxylase	
	4-oxalocrotonate-decarboxylase	Involved in the meta-cleavage pathway for the degradation of phenols, cresols and catechols
	4-hydroxy-4-methyl-2-oxoglutarate aldolase	Also acts on 4-hydroxy-4-methyl-2-oxoadipate and 4-carboxy-4-hydroxy-2-oxohexadioate
	2-oxopent-4-enoate hydratase	Also acts, more slowly, on cis-2-oxohex-4-enoate, but not on the trans-isomer
	Phenylalanine ammonia-lyase	May also act on L-tyrosine
	Phenylalanine racemase (ATP-hydrolysing)	
	Mandelate racemase	
	Phenylpyruvate tautomerase	Also acts on other arylpyruvates
	5-carboxymethyl-2-hydroxymuconate delta-isomerase	
	Muconolactone delta-isomerase	
	Muconate	Also acts, in the reverse reaction, on 3-

	cycloisomerase	methyl-cis-cis-hexa- dienedioate and, very slowly, on cis-trans-hexadienedioate Not identical with EC 5.5.1.7 or EC 5.5.1.11
	3-carboxy-cis,cis-muconate cycloisomerase	
	Carboxy-cis,cis-muconate cyclase	
	Chloromuconate cycloisomerase	Spontaneous elimination of HCl produces cis-4-carboxymethylenebut-2-en-4-olide Also acts in reverse direction on 2-chloro-cis,cis-muconate Not identical with EC 5.5.1.1 or EC 5.5.1.11
	Phenylacetate--CoA ligase	Phenoxyacetate can replace phenylacetate
	Benzoate--CoA ligase	Also acts on 2-, 3- and 4-fluorobenzoate, but only very slowly on the corresponding chlorobenzoates
	4-hydroxybenzoate--CoA ligase	
	Phenylacetate--CoA ligase	Also acts, more slowly, on acetate, propanoate and butanoate, but not on hydroxy derivatives of phenylacetate and related compounds
Phenylalanine, tyrosine and tryptophan biosynthesis	Quinate 5-dehydrogenase	
	Shikimate 5-dehydrogenase	
	Quinate dehydrogenase (pyrroloquinoline-quinone)	
	Phenylalanine 4-monooxygenase	
	Prephenate dehydrogenase	This enzyme in the enteric bacteria also possesses chorismate mutase activity (EC 5.4.99.5) and converts chorismate into prephenate
	Prephenate dehydrogenase (NADP+)	
	Cyclohexadienyl dehydrogenase	Also acts on prephenate and D-prephenyllactate (cf. EC 1.3.1.12)
	2-methyl-branched-	From Ascaris suum The reaction

	chain-enoyl-CoA reductase	proceeds only in the presence of another flavoprotein (ETF=[PRIME]Electron-Transferring Flavoprotein[PRIME])
	Phenylalanine dehydrogenase	The enzyme from <i>Bacillus badius</i> and <i>Sporosarcina ureae</i> are highly specific for L-phenylalanine, that from <i>Bacillus sphaericus</i> also acts on L-tyrosine
	L-amino acid oxidase	
	Antranilate phosphoribosyl-transferase	In some organisms, this enzyme is part of a multifunctional protein together with one or more components of the system for biosynthesis of tryptophan (EC 4.1.1.48, EC 4.1.3.27, EC 4.2.1.20, and EC 5.3.1.24)
	3-phosphoshikimate 1-carboxyvinyl-transferase	
	Aspartate aminotransferase	Also acts on L-tyrosine, L-phenylalanine and L-tryptophan. This activity can be formed from EC 2.6.1.57 by controlled proteolysis
	Tyrosine aminotransferase	L-phenylalanine can act instead of L-tyrosine The mitochondrial enzyme may be identical with EC 2.6.1.1 The three isoenzymic forms are interconverted by EC 3.4.22.4
	Aromatic amino acid transferase	L-methionine can also act as donor, more slowly Oxaloacetate can act as acceptor Controlled proteolysis converts the enzyme to EC 2.6.1.1
	Histidinol-phosphate aminotransferase	
	Shikimate kinase	
	Indole-3-glycerol-phosphate synthase	In some organisms, this enzyme is part of a multifunctional protein together with one or more components of the system for biosynthesis of tryptophan (EC 2.4.2.18, EC 4.1.3.27, EC 4.2.1.20, and EC 5.3.1.24)
	2-dehydro-3-deoxyphosphoheptonate aldolase	
	Anthranilate synthase	In some organisms, this enzyme is part of a multifunctional protein together with

		one or more components of the system for biosynthesis of tryptophan (EC 2.4.2.18, EC 4.1.1.48, EC 4.2.1.20, and EC 5.3.1.24) The native enzyme in the complex with uses either glutamine or (less efficiently) NH(3). The enzyme separated from the complex uses NH(3) only
	3-dehydroquinate dehydratase	
	Phosphopyruvate hydratase	Also acts on 3-phospho-D-erythronate
	Tryptophan synthase	Also catalyses the conversion of serine and indole into tryptophan and water and of indoleglycerol phosphate into indole and glyceraldehyde phosphate In some organisms, this enzyme is part of a multifunctional protein together with one or more components of the system for biosynthesis of tryptophan (EC 2.4.2.18, EC 4.1.1.48, EC 4.1.3.27, and EC 5.3.1.24)
	Prephenate dehydratase	This enzyme in the enteric bacteria also possesses chorismate mutase activity and converts chorismate into prephenate
	Carboxycyclohexadienyl dehydratase	Also acts on prephenate and D-prephenyllactate Cf. EC 4.2.1.51
	3-dehydroquinate synthase	The hydrogen atoms on C-7 of the substrate are retained on C-2 of the products
	Chorismate synthase	Shikimate is numbered so that the double-bond is between C-1 and C-2, but some earlier papers numbered in the reverse direction
	Phosphoribosylanthranilate isomerase	In some organisms, this enzyme is part of a multifunctional protein together with one or more components of the system for biosynthesis of tryptophan (EC 2.4.2.18, EC 4.1.1.48, EC 4.1.3.27, and EC 4.2.1.20)
	Chorismate mutase	
	Tyrosine--tRNA ligase	
	Phenylalanine--tRNA ligase	

Starch and sucrose metabolism	UDP-glucose 6-dehydrogenase	Also acts on UDP-2-deoxyglucose
	Glucoside 3-dehydrogenase	The enzyme acts on D-glucose, D-galactose, D-glucosides and D-galactosides, but D-glucosides react more rapidly than D-galactosides
	CDP-4-dehydro-6-deoxyglucose reductase	Two proteins are involved but no partial reaction has been observed in the presence of either alone
	Phosphorylase	The recommended name should be qualified in each instance by adding the name of the natural substance, e.g. maltodextrin phosphorylase, starch phosphorylase, glycogen phosphorylase
	Levansucrase	Some other sugars can act as D-fructosyl acceptors
	Glycogen (starch) synthase	The recommended name varies according to the source of the enzyme and the nature of its synthetic product Glycogen synthase from animal tissues is a complex of a catalytic subunit and the protein glycogenin. The enzyme requires glucosylated glycogenin as a primer; this is the reaction product of EC 2.4.1.186. A similar enzyme utilizes ADP-glucose (Cf. EC 2.4.1.21)
	Cellulose synthase (UDP-forming)	Involved in the synthesis of cellulose A similar enzyme utilizes GDP-glucose (Cf. EC 2.4.1.29)
	Sucrose synthase	
	Sucrose-phosphate synthase	
	Alpha,alpha-trehalose-phosphate synthase (UDP-forming)	See also EC 2.4.1.36
	UDP-glucuronosyltransferase	Family of enzymes accepting a wide range of substrates, including phenols, alcohols, amines and fatty acids. Some of the activities catalysed were previously listed separately as EC 2.4.1.42, EC 2.4.1.59, EC 2.4.1.61, EC 2.4.1.76, EC 2.4.1.77, EC 2.4.1.84, EC 2.4.1.107 and EC 2.4.1.108. A temporary nomenclature for the various forms whose delineation

		is in a state of flux
	1,4-alpha-glucan branching enzyme	Converts amylose into amylopectin The recommended name requires a qualification depending on the product, glycogen or amylopectin, e.g. glycogen branching enzyme, amylopectin branching enzyme. The latter has frequently been termed Q-enzyme
	Cellobiose phosphorylase	
	Starch (bacterial glycogen) synthase	The recommended name various according to the source of the enzyme and the nature of its synthetic product, e.g. starch synthase, bacterial glycogen synthase A similar enzyme utilizes UDP-glucose (Cf. EC 2.4.1.11)
	4-alpha-glucanotransferase	An enzymic activity of this nature forms part of the mammalian and Yeast glycogen branching system (see EC 3.2.1.33)
	Cellulose synthase (GDP-forming)	Involved in the synthesis of cellulose A similar enzyme utilizes UDP-glucose (Cf. EC 2.4.1.12)
	1,3-beta-glucan synthase	
	Phenol beta-glucosyltransferase	Acts on a wide range of phenols
	Amylosucrase	
	Polygalacturonate 4-alpha-galacturonosyltransferase	
	Dextranucrase	
	Alpha,alpha-trehalose phosphorylase	
	Sucrose phosphorylase	In the forward reaction, arsenate may replace phosphate In the reverse reaction various ketoses and L-arabinose may replace D-fructose
	Maltose phosphorylase	
	1,4-beta-D-xylan synthase	
	Hexokinase	D-glucose, D-mannose, D-fructose, sorbitol and D-glucosamine can act as

		acceptors ITP and dATP can act as donors The liver isoenzyme has sometimes been called glucokinase
	Phosphoglucokinase	
	Glucose-1,6-bisphosphate synthase	D-glucose 6-phosphate can act as acceptor, forming D-glucose 1,6-bisphosphate
	Glucokinase	A group of enzymes found in invertebrates and microorganisms highly specific for glucose
	Fructokinase	
	Glucose-1-phosphate phosphodismutase	
	Protein-N(PI)-phosphohistidine-sugar phosphotransferase	Comprises a group of related enzymes The protein substrate is a phosphocarrier protein of low molecular mass (9.5 Kd) A phosphoenzyme intermediate is formed The enzyme translocates the sugar it phosphorylates into bacteria Aldohexoses and their glycosides and alditols are phosphorylated on O-6; fructose and sorbose on O-1 Glycerol and disaccharides are also substrates
	Glucose-1-phosphate adenylyltransferase	
	Glucose-1-phosphate cytidylyltransferase	
	Glucose-1-phosphate guanylyltransferase	Also acts, more slowly, on D-mannose 1-phosphate
	UTP--glucose-1-phosphate uridylyltransferase	
	Pectinesterase	
	Trehalose-phosphatase	
	Sucrose-phosphatase	
	Glucose-6-phosphatase	Wide distribution in animal tissues Also catalyses potent transphosphorylations from carbamoyl phosphate, hexose phosphates, pyrophosphate, phosphoenolpyruvate and nucleoside di- and triphosphates, to D-glucose, D-mannose, 3-methyl-D-glucose, or 2-deoxy-D-glucose (cf. EC 2.7.1.62, EC 2.7.1.79, and EC 3.9.1.1)

	Alpha-amylase	Acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the alpha-configuration
	Oligo-1,6-glucosidase	Also hydrolyses palatinose The enzyme from intestinal mucosa is a single polypeptide chain also catalysing the reaction of EC 3.2.1.48
	Maltose-6[PRIME]-phosphate glucosidase	Hydrolyses a variety of 6-phospho-D-glucosides, including maltose 6-phosphate, alpha[PRIME]alpha-trehalose 6-phosphate, sucrose 6-phosphate and p-nitrophenyl-alpha-D-glucopyranoside 6-phosphate (as a chromogenic substrate) The enzyme is activated by Fe(II), Mn(II), Co(II) and Ni(II). It is rapidly inactivated in air
	Polygalacturonase	
	Beta-amylase	Acts on starch, glycogen and related polysaccharides and oligosaccharides producing beta-maltose by an inversion
	Alpha-glucosidase	Group of enzymes whose specificity is directed mainly towards the exohydrolysis of 1,4-alpha-glucosidic linkages, and that hydrolyse oligosaccharides rapidly, relative to polysaccharides, which are hydrolysed relatively slowly, or not at all The intestinal enzyme also hydrolyses polysaccharides, catalysing the reactions of EC 3.2.1.3, and, more slowly, hydrolyses 1,6-alpha- D-glucose links
	Beta-glucosidase	Wide specificity for beta-D-glucosides. Some examples also hydrolyse one or more of the following: beta-D-galactosides, alpha-L- arabinosides, beta-D-xylosides, and beta-D-fucosides
	Beta-fructofuranosidase	Substrates include sucrose Also catalyses fructotransferase reactions
	Alpha,alpha-trehalase	
	Glucan 1,4-alpha-glucosidase	Most forms of the enzyme can rapidly hydrolyse 1,6-alpha-D-glucosidic bonds when the next bond in sequence is 1,4, and some preparations of this enzyme

		hydrolyse 1,6- and 1,3-alpha-D-glucosidic bonds in other polysaccharides This entry covers all such enzymes acting on polysaccharides more rapidly than on oligosaccharides EC 3.2.1.20 from mammalian intestine can catalyse similar reactions
	Beta-glucuronidase	
	Amylo-1,6-glucosidase	In mammals and yeast this enzyme is linked to a glycosyltransferase similar to EC 2.4.1.25; together these two activities constitute the glycogen debranching system
	Xylan 1,4-beta-xylosidase	Also hydrolyses xylobiose Some other exoglycosidase activities have been found associated with this enzyme in sheep liver
	Glucan endo-1,3-beta-D-glucosidase	Very limited action on mixed-link (1,3-1,4)-beta-D-glucans Hydrolyses laminarin, paramylon and pachymann Different from EC 3.2.1.6
	Cellulase	Will also hydrolyse 1,4-linkages in beta-D-glucans also containing 1,3-linkages
	Sucrose alpha-glucosidase	This enzyme is isolated from intestinal mucosa as a single polypeptide chain also displaying activity towards isomaltose (oligo-1,6-glucosidase, cf. EC 3.2.1.10)
	Cyclomaltodextrinase	Also hydrolyses linear maltodextrin
	Glucan 1,3-beta-glucosidase	Acts on oligosaccharides but very slowly on laminaribiose
	Levanase	
	Galacturan 1,4-alpha-galacturonidase	
	Glucan 1,4-beta-glucosidase	Acts on 1,4-beta-D-glucans and related oligosaccharides Cellobiose is hydrolysed, very slowly
	Cellulose 1,4-beta-cellobiosidase	
	Alpha,alpha-phosphotrehalase	
	ADP-sugar diphosphatase	Has a distinct specificity from the UDP-sugar pyrophosphatase (EC 3.6.1.45)
	Nucleotide pyrophosphatase	Substrates include NAD(+), NADP(+), FAD, CoA and also ATP and ADP

	UDP-glucuronate decarboxylase	
	CDP-glucose 4,6-dehydratase	
	CDP-abequose epimerase	
	UDP-glucuronate 4-epimerase	
	Glucose-6-phosphate isomerase	Also catalyses the anomerization of D-glucose 6-phosphate
	Phosphoglucomutase	Maximum activity is only obtained in the presence of alpha-D-glucose 1,6-bisphosphate. This bisphosphate is an intermediate in the reaction, being formed by transfer of a phosphate residue from the enzyme to the substrate, but the dissociation of bisphosphate from the enzyme complex is much slower than the overall isomerization. Also, more slowly, catalyses the interconversion of 1-phosphate and 6-phosphate isomers of many other alpha-D-hexoses, and the interconversion of alpha-D-ribose 1-phosphate and 5-phosphate
	Beta-phosphoglucomutase	
	Maltose alpha-D-glucosyltransferase	
Tryptophan metabolism	Indole-3-lactate dehydrogenase	
	Indole-3-acetaldehyde reductase (NADH)	
	Indole-3-acetaldehyde reductase (NADPH)	
	3-hydroxyacyl-CoA dehydrogenase	Also oxidizes S-3-hydroxyacyl-N-acylthioethanolamine and S-3-hydroxyacylhydrolipoate. Some enzymes act, more slowly, with NADP(+). Broad specificity to acyl chain-length (cf. EC 1.1.1.211)
	O-aminophenol oxidase	Isophenoxazine may be formed by a secondary condensation from the initial oxidation product
	Catalase	This enzyme can also act as a peroxidase

		(EC 1.11.1.7) for which several organic substances, especially ethanol, can act as a hydrogen donor A manganese protein containing Mn(III) in the resting state, which also belongs here, is often called pseudocatalase Enzymes from some microorganisms, such as <i>Penicillium simplicissimum</i> , which exhibit both catalase and peroxidase activity, have sometimes been referred to as catalase-peroxidase
	7,8-dihydroxykynurename 8,8A-dioxygenase	
	Tryptophan 2,3-dioxygenase	Broad specificity towards tryptamine and derivatives including D- and L-tryptophan, 5-hydroxytryptophan and serotonin
	Indole 2,3-dioxygenase	The enzyme from <i>Jasminum</i> is a flavoprotein containing copper, and forms anthranilate as the final product One enzyme from <i>Tecoma stans</i> is also a flavoprotein containing copper and uses three atoms of oxygen per molecule of indole, to form anthranil (3,4-benzisoxazole) A second enzyme from <i>Tecoma stans</i> , which is not a flavoprotein, uses four atoms of oxygen and forms anthranilate as the final product
	2,3-dihydroxyindole 2,3-dioxygenase	
	Indoleamine-pyrrole 2,3-dioxygenase	Acts on many substituted and unsubstituted indoleamines, including melatonin Involved in the degradation of melatonin
	3-hydroxyanthranilate 3,4-dioxygenase	The product of the reaction spontaneously rearrange to quinolinic acid (quin)
	Tryptophan 2-monoxygenase	
	Tryptophan 2[PRIME]-dioxygenase	Acts on a number of indolyl-3-alkane derivatives, oxidizing the 3-side-chain in the 2[PRIME]-position. Best substrates

		are L-tryptophan and 5-hydroxy-L-tryptophan
	Kynurenine 3-monoxygenase	
	Unspecific monoxygenase	Acts on a wide range of substrates including many xenobiotics, steroids, fatty acids, vitamins and prostaglandins Reactions catalysed include hydroxylation, epoxidation, N-oxidation, sulfoxidation, N-, S- and O-dealkylations, desulfation, deamination, and reduction of azo, nitro, and N-oxide groups
	Anthranilate 3-monoxygenase	
	Tryptophan 5-monoxygenase	Activated by phosphorylation, catalysed by a CA(2+)-activated protein kinase
	Kynurenine 7,8-hydroxylase	
	Aldehyde dehydrogenase (NAD+)	Wide specificity, including oxidation of D-glucuronolactone to D-glucarate
	Aminomuconate-semialdehyde dehydrogenase	Also acts on 2-hydroxymuconate semialdehyde
	Aldehyde oxidase	Also oxidizes quinoline and pyridine derivatives May be identical with EC 1.1.3.22
	Indole-3-acetaldehyde oxidase	Also oxidizes indole-3-aldehyde and acetaldehyde, more slowly
	Oxoglutarate dehydrogenase (lipoamide)	Component of the multienzyme 2-oxoglutarate dehydrogenase complex
	Kynurename-7,8-dihydrodiol dehydrogenase	
	Glutaryl-CoA dehydrogenase	
	L-amino acid oxidase	
	Amine oxidase (flavin-containing)	Acts on primary amines, and usually also on secondary and tertiary amines
	Amine oxidase (copper-containing)	A group of enzymes including those oxidizing primary amines, diamines and histamine One form of EC 1.3.1.15 from rat kidney also catalyses this reaction

	Acetylindoxyl oxidase	
	Acetylserotonin O-methyltransferase	Some other hydroxyindoles also act as acceptor, more slowly
	Indole-3-pyruvate C-methyltransferase	
	Amine N-methyltransferase	A wide range of primary, secondary, and tertiary amines can act as acceptors, including tryptamine, aniline, nicotine and a variety of drugs and other xenobiotics
	Aralkylamine N-acetyltransferase	Narrow specificity towards aralkylamines, including serotonin Not identical with EC 2.3.1.5
	Acetyl-CoA C-acetyltransferase	
	Tryptophan aminotransferase	Also acts on 5-hydroxytryptophan and, to a lesser extent on the phenyl amino acids
	Kynurenine--oxoglutarate aminotransferase	Also acts on 3-hydroxykynurenine
	Thioglucosidase	Has a wide specificity for thioglycosides
	Amidase	
	Formamidase	Also acts, more slowly, on acetamide, propanamide and butanamide
	Arylformamidase	Also acts on other aromatic formylamines
	Nitrilase	Acts on a wide range of aromatic nitriles including (indole-3-yl)-acetonitrile and also on some aliphatic nitriles, and on the corresponding acid amides (cf. EC 4.2.1.84)
	Kynureninase	Also acts on 3[PRIME]-hydroxykynurenine and some other (3-arylcarbonyl)- alanines
	Aromatic-L-amino-acid decarboxylase	Also acts on L-tryptophan, 5-hydroxy-L-tryptophan and dihydroxy- L-phenylalanine (DOPA)
	Phenylpyruvate decarboxylase	Also acts on indole-3-pyruvate
	Aminocarboxymuconate-semialdehyde decarboxylase	The product rearranges non-enzymically to picolinate
	Tryptophanase	Also catalyses the synthesis of tryptophan from indole and serine Also

		catalyses 2,3-elimination and beta-replacement reactions of some indole-substituted tryptophan analogs of L-cysteine, L-serine and other 3-substituted amino acids
	Enoyl-CoA hydratase	Acts in the reverse direction With cis-compounds, yields (3R)-3-hydroxyacyl-CoA (cf. EC 4.2.1.74)
	Nitrile hydratase	Acts on short-chain aliphatic nitriles, converting them into the corresponding acid amides Does not act on these amides or on aromatic nitriles (cf EC 3.5.5.1)
	Tryptophan-tRNA ligase	
Tyrosine metabolism	Alcohol dehydrogenase	Acts on primary or secondary alcohols or hemiacetals The animal, but not the yeast, enzyme acts also on cyclic secondary alcohols
	(R)-4-hydroxyphenyllactate dehydrogenase	Also acts, more slowly, on (R)-3-phenyllactate, (R)-3-(indole-3-yl)lactate and (R)-lactate
	Hydroxyphenylpyruvate reductase	Also acts on 3-(3,4-dihydroxyphenyl)lactate Involved with EC 2.3.1.140 in the biosynthesis of rosmarinic acid
	Aryl-alcohol dehydrogenase	A group of enzymes with broad specificity towards primary alcohols with an aromatic or cyclohex-1-ene ring, but with low or no activity towards short-chain aliphatic alcohols
	Catechol oxidase	Also acts on a variety of substituted catechols Many of these enzymes also catalyse the reaction listed under EC 1.14.18.1; this is especially true for the classical tyrosinase
	Iodide peroxidase	
	3,4-dihydroxyphenylacetate 2,3-dioxygenase	
	4-hydroxyphenylpyruvate dioxygenase	
	Stizolobate synthase	The intermediate product undergoes ring closure and oxidation, with NAD(P)(+)

		as acceptor, to stizolobic acid
	Stizolobinate synthase	The intermediate product undergoes ring closure and oxidation, with NAD(P)(+) as acceptor, to stizolobinic acid
	Gentisate 1,2-dioxygenase	
	Homogentisate 1,2-dioxygenase	
	4-hydroxyphenylacetate 1-monoxygenase	Also acts on 4-hydroxyhydratropate forming 2-methylhomogentisate and on 4-hydroxyphenoxyacetate forming hydroquinone and glycolate
	4-hydroxyphenylacetate 3-monoxygenase	
	Tyrosine N-monoxygenase	
	Hydroxyphenylacetonitrile 2-monoxygenase	
	Tyrosine 3-monoxygenase	Activated by phosphorylation, catalysed by EC 2.7.1.128
	Dopamine-beta-monoxygenase	Stimulated by fumarate
	Monophenol monooxygenase	A group of copper proteins that also catalyse the reaction of EC 1.10.3.1, if only 1,2-benzenediols are available as substrate
	Succinate-semialdehyde dehydrogenase (NAD(P)+)	
	Aryl-aldehyde dehydrogenase	Oxidizes a number of aromatic aldehydes, but not aliphatic aldehydes
	Aldehyde dehydrogenase (NAD+)	Wide specificity, including oxidation of D-glucuronolactone to D-glucarate
	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase	Does not act on unsubstituted aliphatic or aromatic aldehydes or glucose NAD(+) can replace NADP(+), but with lower affinity
	Aldehyde dehydrogenase (NAD(P)+)	
	4-hydroxyphenylacetaldehyde dehydrogenase	With EC 4.2.1.87, brings about the metabolism of octopamine in Pseudomonas

	Aldehyde oxidase	Also oxidizes quinoline and pyridine derivatives May be identical with EC 1.1.3.22
	L-amino acid oxidase	
	Amine oxidase (flavin-containing)	Acts on primary amines, and usually also on secondary and tertiary amines
	Amine oxidase (copper-containing)	A group of enzymes including those oxidizing primary amines, diamines and histamine One form of EC 1.3.1.15 from rat kidney also catalyses this reaction
	Aralkylamine dehydrogenase	Phenazine methosulfate can act as acceptor Acts on aromatic amines and, more slowly, on some long-chain aliphatic amines, but not on methylamine or ethylamine (cf EC 1.4.99.3)
	Phenol O-methyltransferase	Acts on a wide variety of simple alkyl-, methoxy- and halo-phenols
	Tyramine N-methyltransferase	Has some activity on phenylethylamine analogs
	Phenylethanolamine N-methyltransferase	Acts on various phenylethanolamines; converts noradrenalin into adrenalin
	Catechol O-methyltransferase	The mammalian enzymes act more rapidly on catecholamines such as adrenaline or noradrenaline than on catechols
	Glutamine N-phenylacetyltransferase	
	Rosmarinate synthase	Involved with EC 1.1.1.237 in the biosynthesis of rosmarinic acid
	Hydroxymandelonitrile glucosyltransferase	3,4-dihydroxymandelonitrile can also act as acceptor
	Aspartate aminotransferase	Also acts on L-tyrosine, L-phenylalanine and L-tryptophan. This activity can be formed from EC 2.6.1.57 by controlled proteolysis
	Dihydroxyphenylalanine aminotransferase	
	Tyrosine aminotransferase	L-phenylalanine can act instead of L-tyrosine The mitochondrial enzyme may be identical with EC 2.6.1.1 The three isoenzymic forms are interconverted by EC 3.4.22.4
	Aromatic amino acid transferase	L-methionine can also act as donor, more slowly Oxaloacetate can act as acceptor

		Controlled proteolysis converts the enzyme to EC 2.6.1.1
	Histidinol-phosphate aminotransferase	
	Fumarylacetoacetate	Also acts on other 3,5- and 2,4-dioxo acids
	Acylypyruvate hydrolase	Acts on formylpyruvate, 2,4-dioxopentanoate, 2,4-dioxohexanoate and 2,4-dioxoheptanoate
	Tyrosine decarboxylase	The bacterial enzyme also acts on 3-hydroxytyrosine and, more slowly, on 3-hydroxyphenylalanine
	Aromatic-L-amino-acid decarboxylase	Also acts on L-tryptophan, 5-hydroxy-L-tryptophan and dihydroxy- L-phenylalanine (DOPA)
	Gentisate decarboxylase	
	5-oxopent-3-ene-1,2,5-tricarboxylate decarboxylase	
	Tyrosine phenol-lyase	Also slowly catalyses pyruvate formation from D-tyrosine, S-methyl- L-cysteine, L-cysteine, L-serine and D-serine
	(S)-norcoclaurine synthase	The reaction makes a 6-membered ring by forming a bond between C-6 of the 3,4-dihydroxyphenyl group of the dopamine and C-1 of the aldehyde in the imine formed between the substrates. The product is the precursor of the benzylisoquinoline alkaloids in plants. Will also catalyse the reaction of 4-(2-aminoethyl)benzene-1,2-diol + (3,4-dihydroxyphenyl)acetaldehyde to form (S)-norlaudanosoline, but this alkaloid has not been found to occur in plants
	Dihydroxyphenylalanine ammonia-lyase	
	Phenylalanine ammonia-lyase	May also act on L-tyrosine
	Maleylacetoacetate isomerase	Also acts on maleylpyruvate
	Maleylpyruvate isomerase	
	Phenylpyruvate	Also acts on other arylpyruvates

	tautomerase	
	5-carboxymethyl-2-hydroxymuconate delta-isomerase	
	Tyrosine 2,3-aminomutase	
	Phenylacetate--CoA ligase	Also acts, more slowly, on acetate, propanoate and butanoate, but not on hydroxy derivatives of phenylacetate and related compounds

## VII. PROMOTERS AS SENTINELS

Useful promoters include those that are capable of facilitating preferential transcription, i.e. tissue-specific or developmentally regulated gene expression and being a component of facile systems to evaluate the metabolic/physiological state of a plant cell, tissue or organ. Many such promoters are included in this application. Operably linking a sequence to these promoters that can act as a reporter and inserting the construct into a plant allows detection of the preferential in plantar transcription. For example, the quantitative state of responses to environmental conditions can be detected by using a plant having a construct that contains a stress-inducible promoter linked to and controlling expression of a sequence encoding GFP. The greater the stress promoter is induced, the greater the levels of fluorescence from GFP will be produced and this provides a measure of the level of stress being expressed by the plant and/or the ability of the plant to respond internally to the stress.

More specifically, using this system the activities of any metabolic pathway (catabolic and anabolic), stress-related pathways as on any plant gene repeated activity can be monitored. In addition, assays can be developed using this sentinel system to select for superior genotypes with greater yield characteristics or to select for plants with altered responses to chemical, herbicide, or plant growth regulators or to identify chemical, herbicides or plant growth regulators by their response on such sentinels.

Specifically, a promoter that is regulated in plants in the desired way, is operably linked to a reporter such as GFP, RFP, etc., and the constructs are introduced into the plant of interest. The behavior of the reporter is monitored using technologies typically specific for that reporter. With GFP, RFP, etc., it could typically be by microscopy of whole plants, organs, tissues or cells under excitation by an appropriate wavelength of UV light.

## **VIII. HOW TO MAKE DIFFERENT EMBODIMENTS OF THE INVENTION**

The invention relates to (I) polynucleotides and methods of use thereof, such as

- IA. Probes, Primers and Substrates;
- IB. Methods of Detection and Isolation;
  - B.1. Hybridization;
  - B.2. Methods of Mapping;
  - B.3. Southern Blotting;
  - B.4. Isolating cDNA from Related Organisms;
  - B.5. Isolating and/or Identifying Orthologous Genes
- IC. Methods of Inhibiting Gene Expression
  - C.1. Antisense
  - C.2. Ribozyme Constructs;
  - C.3. Chimeraplasts;
  - C.4. Co-Suppression;
  - C.5. Transcriptional Silencing
  - C.6. Other Methods to Inhibit Gene Expression
- ID. Methods of Functional Analysis;
- IE. Promoter Sequences and Their Use;
- IF. UTRs and/or Intron Sequences and Their Use; and
- IG. Coding Sequences and Their Use.

The invention also relates to (II) polypeptides and proteins and methods of use thereof, such

as

- IIA. Native Polypeptides and Proteins
  - A.1 Antibodies
  - A.2 In Vitro Applications
- IIB. Polypeptide Variants, Fragments and Fusions
  - B.1 Variants
  - B.2 Fragments
  - B.3 Fusions

The invention also includes (III) methods of modulating polypeptide production, such as

IIIA. Suppression

- A.1 Antisense
- A.2 Ribozymes
- A.3 Co-suppression
- A.4 Insertion of Sequences into the Gene to be Modulated
- A.5 Promoter Modulation
- A.6 Expression of Genes containing Dominant-Negative Mutations

IIIB. Enhanced Expression

- B.1 Insertion of an Exogenous Gene
- B.2 Promoter Modulation

The invention further concerns (IV) gene constructs and vector construction, such as

IVA. Coding Sequences

IVB. Promoters

IVC. Signal Peptides

The invention still further relates to

V. Transformation Techniques

I. Polynucleotides

Exemplified SDFs of the invention represent fragments of the genome of corn, wheat, rice, soybean or *Arabidopsis* and/or represent mRNA expressed from that genome. The isolated nucleic acid of the invention also encompasses corresponding fragments of the genome and/or cDNA complement of other organisms as described in detail below.

Polynucleotides of the invention can be isolated from polynucleotide libraries using primers comprising sequences similar to those described, in the attached Reference, Sequences Protein Group, and Protein Group Matrix Tables or complements thereof. See, for example, the methods described in Sambrook *et al., supra*.

Alternatively, the polynucleotides of the invention can be produced by chemical synthesis. Such synthesis methods are described below.

It is contemplated that the nucleotide sequences presented herein may contain some small percentage of errors. These errors may arise in the normal course of determination of nucleotide sequences. Sequence errors can be corrected by obtaining seeds deposited under the accession numbers cited above, propagating them, isolating genomic DNA or appropriate mRNA from the resulting plants or seeds thereof, amplifying the relevant fragment of the genomic DNA or mRNA using primers having a sequence that flanks the erroneous sequence, and sequencing the amplification product.

#### I.A. Probes, Primers and Substrates

SDFs of the invention can be applied to substrates for use in array applications such as, but not limited to, assays of global gene expression, for example under varying conditions of development, growth conditions. The arrays can also be used in diagnostic or forensic methods (WO95/35505, US 5,445,943 and US 5,410,270).

Probes and primers of the instant invention will hybridize to a polynucleotide comprising a sequence in or encoded by those in the Reference, Sequence, Protein Group, and Protein Group Matrix tables or fragments or complement thereof. Though many different nucleotide sequences can encode an amino acid sequence, the sequences of the reference and Sequence table or sequences that encode polypeptides or fragments thereof described in Protein Group and Protein Group Matrix tables are generally preferred for encoding polypeptides of the invention.

However, the sequence of the probes and/or primers of the instant invention need not be identical to those in the Reference and Sequence tables or the complements thereof. For example, some variation in probe or primer sequence and/or length can allow additional family members to be detected, as well as orthologous genes and more taxonomically distant related sequences.

Similarly, probes and/or primers of the invention can include additional nucleotides that serve as a label for detecting the formed duplex or for subsequent cloning purposes.

Probe length will vary depending on the application. For use as primers, probes are 12-40 nucleotides, preferably 18-30 nucleotides long. For use in mapping, probes are preferably 50

to 500 nucleotides, preferably 100-250 nucleotides long. For Southern hybridizations, probes as long as several kilobases can be used as explained below.

The probes and/or primers can be produced by synthetic procedures such as the triester method of Matteucci et al. *J. Am. Chem. Soc.* 103:3185( 1981); or according to Urdea et al. *Proc. Natl. Acad. Sci. U.S.A.* 80:7461 (1981) or using commercially available automated oligonucleotide synthesizers.

#### I.B. Methods of Detection and Isolation

The polynucleotides of the invention can be utilized in a number of methods known to those skilled in the art as probes and/or primers to isolate and detect polynucleotides, including, without limitation: Southern, Northern, Branched DNA hybridization assays, polymerase chain reaction, and microarray assays, and variations thereof. Specific methods given by way of examples, and discussed below include:

Hybridization

Methods of Mapping

Southern Blotting

Isolating cDNA from Related Organisms

Isolating and/or Identifying Orthologous Genes.

Also, the nucleic acid molecules of the invention can be used in other methods, such as high density oligonucleotide hybridizing assays, described, for example, in U.S. Pat. Nos. 6,004,753; 5,945,306; 5,945,287; 5,945,308; 5,919,686; 5,919,661; 5,919,627; 5,874,248; 5,871,973; 5,871,971; and 5,871,930; and PCT Pub. Nos. WO 9946380; WO 9933981; WO 9933870; WO 9931252; WO 9915658; WO 9906572; WO 9858052; WO 9958672; and WO 9810858.

#### B.1. Hybridization

The isolated SDFs of the Reference and Sequence tables or SDFs encoding polypeptides of the Protein Group and Protein Group Matrix tables or fragments thereof of the present invention can be used as probes and/or primers for detection and/or isolation of related polynucleotide sequences through hybridization. Hybridization of one nucleic acid to another constitutes a physical property that defines the subject SDF of the invention and the identified

related sequences. Also, such hybridization imposes structural limitations on the pair. A good general discussion of the factors for determining hybridization conditions is provided by Sambrook et al. ("Molecular Cloning, a Laboratory Manual, 2nd ed., c. 1989 by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; *see esp.*, chapters 11 and 12). Additional considerations and details of the physical chemistry of hybridization are provided by G.H. Keller and M.M. Manak "DNA Probes", 2<sup>nd</sup> Ed. pp. 1-25, c. 1993 by Stockton Press, New York, NY.

Depending on the stringency of the conditions under which these probes and/or primers are used, polynucleotides exhibiting a wide range of similarity to those in the Reference and Sequence or encoding polypeptides of the Protein Group and Protein Group Matrix tables or fragments thereof can be detected or isolated. When the practitioner wishes to examine the result of membrane hybridizations under a variety of stringencies, an efficient way to do so is to perform the hybridization under a low stringency condition, then to wash the hybridization membrane under increasingly stringent conditions.

When using SDFs to identify orthologous genes in other species, the practitioner will preferably adjust the amount of target DNA of each species so that, as nearly as is practical, the same number of genome equivalents are present for each species examined. This prevents faint signals from species having large genomes, and thus small numbers of genome equivalents per mass of DNA, from erroneously being interpreted as absence of the corresponding gene in the genome.

The probes and/or primers of the instant invention can also be used to detect or isolate nucleotides that are "identical" to the probes or primers. Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below.

Isolated polynucleotides within the scope of the invention also include allelic variants of the specific sequences presented in the Reference, Sequence, Protein Group, and Protein Group Matrix tables. The probes and/or primers of the invention can also be used to detect and/or isolate polynucleotides exhibiting at least 80% sequence identity with the sequences of the reference, Sequence or encoding polypeptides of the Protein Group and Protein Group Matrix tables or fragments thereof.

With respect to nucleotide sequences, degeneracy of the genetic code provides the possibility to substitute at least one base of the base sequence of a gene with a different base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, the DNA of the present invention may also have any base sequence that has been changed from a sequence in the Reference, Sequence, Protein Group, and Protein Group Matrix tables by substitution in accordance with degeneracy of genetic code. References describing codon usage include: Carels *et al.*, *J. Mol. Evol.* **46**: 45 (1998) and Fennoy *et al.*, *Nucl. Acids Res.* **21**(23): 5294 (1993).

#### B.2. Mapping

The isolated SDF DNA of the invention can be used to create various types of genetic and physical maps of the genome of corn, Arabidopsis, soybean, rice, wheat, or other plants. Some SDFs may be absolutely associated with particular phenotypic traits, allowing construction of gross genetic maps. While not all SDFs will immediately be associated with a phenotype, all SDFs can be used as probes for identifying polymorphisms associated with phenotypes of interest. Briefly, one method of mapping involves total DNA isolation from individuals. It is subsequently cleaved with one or more restriction enzymes, separated according to mass, transferred to a solid support, hybridized with SDF DNA and the pattern of fragments compared. Polymorphisms associated with a particular SDF are visualized as differences in the size of fragments produced between individual DNA samples after digestion with a particular restriction enzyme and hybridization with the SDF. After identification of polymorphic SDF sequences, linkage studies can be conducted. By using the individuals showing polymorphisms as parents in crossing programs, F2 progeny recombinants or recombinant inbreds, for example, are then analyzed. The order of DNA polymorphisms along the chromosomes can be determined based on the frequency with which they are inherited together versus independently. The closer two polymorphisms are together in a chromosome the higher the probability that they are inherited together. Integration of the relative positions of all the polymorphisms and associated marker SDFs can produce a genetic map of the species, where the distances between markers reflect the recombination frequencies in that chromosome segment.

The use of recombinant inbred lines for such genetic mapping is described for *Arabidopsis* by Alonso-Blanco et al. (*Methods in Molecular Biology*, vol.82, "Arabidopsis Protocols", pp. 137-146, J.M. Martinez-Zapater and J. Salinas, eds., c. 1998 by Humana Press, Totowa, NJ) and for corn by Burr ("Mapping Genes with Recombinant Inbreds", pp. 249-254. In Freeling, M. and V. Walbot (Ed.), *The Maize Handbook*, c. 1994 by Springer-Verlag New York, Inc.: New York, NY, USA; Berlin Germany; Burr et al. *Genetics* (1998) 118: 519; Gardiner, J. et al., (1993) *Genetics* 134: 917). This procedure, however, is not limited to plants and can be used for other organisms (such as yeast) or for individual cells.

The SDFs of the present invention can also be used for simple sequence repeat (SSR) mapping. Rice SSR mapping is described by Morgante et al. (*The Plant Journal* (1993) 3: 165), Panaud et al. (*Genome* (1995) 38: 1170); Senior et al. (*Crop Science* (1996) 36: 1676), Taramino et al. (*Genome* (1996) 39: 277) and Ahn et al. (*Molecular and General Genetics* (1993) 241: 483-90). SSR mapping can be achieved using various methods. In one instance, polymorphisms are identified when sequence specific probes contained within an SDF flanking an SSR are made and used in polymerase chain reaction (PCR) assays with template DNA from two or more individuals of interest. Here, a change in the number of tandem repeats between the SSR-flanking sequences produces differently sized fragments (U.S. Patent 5,766,847). Alternatively, polymorphisms can be identified by using the PCR fragment produced from the SSR-flanking sequence specific primer reaction as a probe against Southern blots representing different individuals (U.H. Refseth et al., (1997) *Electrophoresis* 18: 1519).

Genetic and physical maps of crop species have many uses. For example, these maps can be used to devise positional cloning strategies for isolating novel genes from the mapped crop species. In addition, because the genomes of closely related species are largely syntenic (that is, they display the same ordering of genes within the genome), these maps can be used to isolate novel alleles from relatives of crop species by positional cloning strategies.

The various types of maps discussed above can be used with the SDFs of the invention to identify Quantitative Trait Loci (QTLs). Many important crop traits, such as the solids content of tomatoes, are quantitative traits and result from the combined interactions of several genes. These genes reside at different loci in the genome, oftentimes on different chromosomes, and generally exhibit multiple alleles at each locus. The SDFs of the invention can be used to

identify QTLs and isolate specific alleles as described by de Vicente and Tanksley (*Genetics* 134:585 (1993)). In addition to isolating QTL alleles in present crop species, the SDFs of the invention can also be used to isolate alleles from the corresponding QTL of wild relatives. Transgenic plants having various combinations of QTL alleles can then be created and the effects of the combinations measured. Once a desired allele combination has been identified, crop improvement can be accomplished either through biotechnological means or by directed conventional breeding programs (for review see Tanksley and McCouch, *Science* 277:1063 (1997)).

In another embodiment, the SDFs can be used to help create physical maps of the genome of corn, *Arabidopsis* and related species. Where SDFs have been ordered on a genetic map, as described above, they can be used as probes to discover which clones in large libraries of plant DNA fragments in YACs, BACs, etc. contain the same SDF or similar sequences, thereby facilitating the assignment of the large DNA fragments to chromosomal positions. Subsequently, the large BACs, YACs, etc. can be ordered unambiguously by more detailed studies of their sequence composition (e.g. Marra et al. (1997) *Genomic Research* 7:1072-1084) and by using their end or other sequences to find the identical sequences in other cloned DNA fragments. The overlapping of DNA sequences in this way allows large contigs of plant sequences to be built that, when sufficiently extended, provide a complete physical map of a chromosome. Sometimes the SDFs themselves will provide the means of joining cloned sequences into a contig.

The patent publication WO95/35505 and U.S. Patents 5,445,943 and 5,410,270 describe scanning multiple alleles of a plurality of loci using hybridization to arrays of oligonucleotides. These techniques are useful for each of the types of mapping discussed above.

Following the procedures described above and using a plurality of the SDFs of the present invention, any individual can be genotyped. These individual genotypes can be used for the identification of particular cultivars, varieties, lines, ecotypes and genetically modified plants or can serve as tools for subsequent genetic studies involving multiple phenotypic traits.

### B.3 Southern Blot Hybridization

The sequences from Reference and Sequence and those encoding polypeptides of Protein Group and Protein Group Matrix tables or fragments thereof can be used as probes for various

hybridization techniques. These techniques are useful for detecting target polynucleotides in a sample or for determining whether transgenic plants, seeds or host cells harbor a gene or sequence of interest and thus might be expected to exhibit a particular trait or phenotype.

In addition, the SDFs from the invention can be used to isolate additional members of gene families from the same or different species and/or orthologous genes from the same or different species. This is accomplished by hybridizing an SDF to, for example, a Southern blot containing the appropriate genomic DNA or cDNA. Given the resulting hybridization data, one of ordinary skill in the art could distinguish and isolate the correct DNA fragments by size, restriction sites, sequence and stated hybridization conditions from a gel or from a library.

Identification and isolation of orthologous genes from closely related species and alleles within a species is particularly desirable because of their potential for crop improvement. Many important crop traits, such as the solid content of tomatoes, result from the combined interactions of the products of several genes residing at different loci in the genome. Generally, alleles at each of these loci can make quantitative differences to the trait. By identifying and isolating numerous alleles for each locus from within or different species, transgenic plants with various combinations of alleles can be created and the effects of the combinations measured. Once a more favorable allele combination has been identified, crop improvement can be accomplished either through biotechnological means or by directed conventional breeding programs (Tanksley et al. *Science* 277:1063(1997)).

The results from hybridizations of the SDFs of the invention to, for example, Southern blots containing DNA from another species can also be used to generate restriction fragment maps for the corresponding genomic regions. These maps provide additional information about the relative positions of restriction sites within fragments, further distinguishing mapped DNA from the remainder of the genome.

Physical maps can be made by digesting genomic DNA with different combinations of restriction enzymes.

Probes for Southern blotting to distinguish individual restriction fragments can range in size from 15 to 20 nucleotides to several thousand nucleotides. More preferably, the probe is 100 to 1,000 nucleotides long for identifying members of a gene family when it is found that repetitive sequences would complicate the hybridization. For identifying an entire corresponding gene in

another species, the probe is more preferably the length of the gene, typically 2,000 to 10,000 nucleotides, but probes 50-1,000 nucleotides long might be used. Some genes, however, might require probes up to 1,500 nucleotides long or overlapping probes constituting the full-length sequence to span their lengths.

Also, while it is preferred that the probe be homogeneous with respect to its sequence, it is not necessary. For example, as described below, a probe representing members of a gene family having diverse sequences can be generated using PCR to amplify genomic DNA or RNA templates using primers derived from SDFs that include sequences that define the gene family.

For identifying corresponding genes in another species, the next most preferable probe is a cDNA spanning the entire coding sequence, which allows all of the mRNA-coding fragment of the gene to be identified. Probes for Southern blotting can easily be generated from SDFs by making primers having the sequence at the ends of the SDF and using corn or *Arabidopsis* genomic DNA as a template. In instances where the SDF includes sequence conserved among species, primers including the conserved sequence can be used for PCR with genomic DNA from a species of interest to obtain a probe.

Similarly, if the SDF includes a domain of interest, that fragment of the SDF can be used to make primers and, with appropriate template DNA, used to make a probe to identify genes containing the domain. Alternatively, the PCR products can be resolved, for example by gel electrophoresis, and cloned and/or sequenced. Using Southern hybridization, the variants of the domain among members of a gene family, both within and across species, can be examined.

#### B.4.1 Isolating DNA from Related Organisms

The SDFs of the invention can be used to isolate the corresponding DNA from other organisms. Either cDNA or genomic DNA can be isolated. For isolating genomic DNA, a lambda, cosmid, BAC or YAC, or other large insert genomic library from the plant of interest can be constructed using standard molecular biology techniques as described in detail by Sambrook et al. 1989 (*Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory Press, New York) and by Ausubel et al. 1992 (*Current Protocols in Molecular Biology*, Greene Publishing, New York).

To screen a phage library, for example, recombinant lambda clones are plated out on appropriate bacterial medium using an appropriate *E. coli* host strain. The resulting plaques are lifted from the plates using nylon or nitrocellulose filters. The plaque lifts are processed through denaturation, neutralization, and washing treatments following the standard protocols outlined by Ausubel et al. (1992). The plaque lifts are hybridized to either radioactively labeled or non-radioactively labeled SDF DNA at room temperature for about 16 hours, usually in the presence of 50% formamide and 5X SSC (sodium chloride and sodium citrate) buffer and blocking reagents. The plaque lifts are then washed at 42°C with 1% Sodium Dodecyl Sulfate (SDS) and at a particular concentration of SSC. The SSC concentration used is dependent upon the stringency at which hybridization occurred in the initial Southern blot analysis performed. For example, if a fragment hybridized under medium stringency (e.g., Tm - 20°C), then this condition is maintained or preferably adjusted to a less stringent condition (e.g., Tm-30°C) to wash the plaque lifts. Positive clones show detectable hybridization e.g., by exposure to X-ray films or chromogen formation. The positive clones are then subsequently isolated for purification using the same general protocol outlined above. Once the clone is purified, restriction analysis can be conducted to narrow the region corresponding to the gene of interest. The restriction analysis and succeeding subcloning steps can be done using procedures described by, for example Sambrook et al. (1989) cited above.

The procedures outlined for the lambda library are essentially similar to those used for YAC library screening, except that the YAC clones are harbored in bacterial colonies. The YAC clones are plated out at reasonable density on nitrocellulose or nylon filters supported by appropriate bacterial medium in petri plates. Following the growth of the bacterial clones, the filters are processed through the denaturation, neutralization, and washing steps following the procedures of Ausubel et al. 1992. The same hybridization procedures for lambda library screening are followed.

To isolate cDNA, similar procedures using appropriately modified vectors are employed. For instance, the library can be constructed in a lambda vector appropriate for cloning cDNA such as  $\lambda$ gt11. Alternatively, the cDNA library can be made in a plasmid vector. cDNA for cloning can be prepared by any of the methods known in the art, but is preferably prepared as described above. Preferably, a cDNA library will include a high proportion of full-length clones.

B. 5. Isolating and/or Identifying Orthologous Genes

Probes and primers of the invention can be used to identify and/or isolate polynucleotides related to those in the Reference, Sequence, Protein Group, and Protein Group Matrix tables. Related polynucleotides are those that are native to other plant organisms and exhibit either similar sequence or encode polypeptides with similar biological activity. One specific example is an orthologous gene. Orthologous genes have the same functional activity. As such, orthologous genes may be distinguished from homologous genes. The percentage of identity is a function of evolutionary separation and, in closely related species, the percentage of identity can be 98 to 100%. The amino acid sequence of a protein encoded by an orthologous gene can be less than 75% identical, but tends to be at least 75% or at least 80% identical, more preferably at least 90%, most preferably at least 95% identical to the amino acid sequence of the reference protein.

To find orthologous genes, the probes are hybridized to nucleic acids from a species of interest under low stringency conditions, preferably one where sequences containing as much as 40-45% mismatches will be able to hybridize. This condition is established by  $T_m - 40^{\circ}\text{C}$  to  $T_m - 48^{\circ}\text{C}$  (see below). Blots are then washed under conditions of increasing stringency. It is preferable that the wash stringency be such that sequences that are 85 to 100% identical will hybridize. More preferably, sequences 90 to 100% identical will hybridize and most preferably only sequences greater than 95% identical will hybridize. One of ordinary skill in the art will recognize that, due to degeneracy in the genetic code, amino acid sequences that are identical can be encoded by DNA sequences as little as 67% identical or less. Thus, it is preferable, for example, to make an overlapping series of shorter probes, on the order of 24 to 45 nucleotides, and individually hybridize them to the same arrayed library to avoid the problem of degeneracy introducing large numbers of mismatches.

As evolutionary divergence increases, genome sequences also tend to diverge. Thus, one of skill will recognize that searches for orthologous genes between more divergent species will require the use of lower stringency conditions compared to searches between closely related species. Also, degeneracy of the genetic code is more of a problem for searches in the genome of a species more distant evolutionarily from the species that is the source of the SDF probe sequences.

Therefore the method described in Bouckaert et al., U.S. Ser. No. 60/121,700 Atty. Dkt. No. 2750-117P, Client Dkt. No. 00010.001, filed February 25, 1999, hereby incorporated in its entirety by reference, can be applied to the SDFs of the present invention to isolate related genes from plant species which do not hybridize to the corn *Arabidopsis*, soybean, rice, wheat, and other plant sequences of the reference, Sequence, Protein Group, and Protein Group Matrix tables.

Identification of the relationship of nucleotide or amino acid sequences among plant species can be done by comparing the nucleotide or amino acid sequences of SDFs of the present application with nucleotide or amino acid sequences of other SDFs such as those present in applications listed in the table below:

The SDFs of the invention can also be used as probes to search for genes that are related to the SDF within a species. Such related genes are typically considered to be members of a gene family. In such a case, the sequence similarity will often be concentrated into one or a few fragments of the sequence. The fragments of similar sequence that define the gene family typically encode a fragment of a protein or RNA that has an enzymatic or structural function. The percentage of identity in the amino acid sequence of the domain that defines the gene family is preferably at least 70%, more preferably 80 to 95%, most preferably 85 to 99%. To search for members of a gene family within a species, a low stringency hybridization is usually performed, but this will depend upon the size, distribution and degree of sequence divergence of domains that define the gene family. SDFs encompassing regulatory regions can be used to identify coordinately expressed genes by using the regulatory region sequence of the SDF as a probe.

In the instances where the SDFs are identified as being expressed from genes that confer a particular phenotype, then the SDFs can also be used as probes to assay plants of different species for those phenotypes.

#### I.C. Methods to Inhibit Gene Expression

The nucleic acid molecules of the present invention can be used to inhibit gene transcription and/or translation. Examples of such methods include, without limitation:

Antisense Constructs;

Ribozyme Constructs;

Chimeroplast Constructs;  
Co-Suppression;  
Transcriptional Silencing; and  
Other Methods of Gene Expression.

C.1 Antisense

In some instances it is desirable to suppress expression of an endogenous or exogenous gene. A well-known instance is the FLAVOR-SAVOR™ tomato, in which the gene encoding ACC synthase is inactivated by an antisense approach, thus delaying softening of the fruit after ripening. See for example, U.S. Patent No. 5,859,330; U.S. Patent No. 5,723,766; Oeller, et al, *Science*, 254:437-439(1991); and Hamilton et al, *Nature*, 346:284-287 (1990). Also, timing of flowering can be controlled by suppression of the *FLOWERING LOCUS C (FLC)*; high levels of this transcript are associated with late flowering, while absence of *FLC* is associated with early flowering (S.D. Michaels et al., *Plant Cell* 11:949 (1999)). Also, the transition of apical meristem from production of leaves with associated shoots to flowering is regulated by *TERMINAL FLOWER1*, *APETALA1* and *LEAFY*. Thus, when it is desired to induce a transition from shoot production to flowering, it is desirable to suppress *TFL1* expression (S.J. Liljegren, *Plant Cell* 11:1007 (1999)). As another instance, arrested ovule development and female sterility result from suppression of the ethylene forming enzyme but can be reversed by application of ethylene (D. De Martinis et al., *Plant Cell* 11:1061 (1999)). The ability to manipulate female fertility of plants is useful in increasing fruit production and creating hybrids.

In the case of polynucleotides used to inhibit expression of an endogenous gene, the introduced sequence need not be perfectly identical to a sequence of the target endogenous gene. The introduced polynucleotide sequence will typically be at least substantially identical to the target endogenous sequence.

Some polynucleotide SDFs in the Reference, Sequence, Protein Group, and Protein Group Matrix tables represent sequences that are expressed in corn, wheat, rice, soybean *Arabidopsis* and/or other plants. Thus the invention includes using these sequences to generate antisense constructs to inhibit translation and/or degradation of transcripts of said SDFs, typically in a plant cell.

To accomplish this, a polynucleotide segment from the desired gene that can hybridize to the mRNA expressed from the desired gene (the "antisense segment") is operably linked to a promoter such that the antisense strand of RNA will be transcribed when the construct is present in a host cell. A regulated promoter can be used in the construct to control transcription of the antisense segment so that transcription occurs only under desired circumstances.

The antisense segment to be introduced generally will be substantially identical to at least a fragment of the endogenous gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. Further, the antisense product may hybridize to the untranslated region instead of or in addition to the coding sequence of the gene. The vectors of the present invention can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene.

For antisense suppression, the introduced antisense segment sequence also need not be full length relative to either the primary transcription product or the fully processed mRNA. Generally, a higher percentage of sequence identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and the full length of the transcript canbe used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 500 nucleotides is especially preferred.

### C.2. Ribozymes

It is also contemplated that gene constructs representing ribozymes and based on the SDFs in the Reference and Sequence tables or those encoding polypeptides of the Protein Group and Protein Group Matrix tables and fragment thereof are an object of the invention. Ribozymes can also be used to inhibit expression of genes by suppressing the translation of the mRNA into a polypeptide. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The inclusion of ribozyme

sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs, which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Haseloff et al. *Nature*, 334:585 (1988).

Like the antisense constructs above, the ribozyme sequence fragment necessary for pairing need not be identical to the target nucleotides to be cleaved, nor identical to the sequences in the Reference and Sequence tables or those encoding polypeptide of the Protein Group and Protein Group Matrix tables or fragments thereof. Ribozymes may be constructed by combining the ribozyme sequence and some fragment of the target gene which would allow recognition of the target gene mRNA by the resulting ribozyme molecule. Generally, the sequence in the ribozyme capable of binding to the target sequence exhibits a percentage of sequence identity with at least 80%, preferably with at least 85%, more preferably with at least 90% and most preferably with at least 95%, even more preferably, with at least 96%, 97%, 98% or 99% sequence identity to some fragment of a sequence in the Reference, Sequence, Protein Group, and Protein Group Matrix tables or the complement thereof. The ribozyme can be equally effective in inhibiting mRNA translation by cleaving either in the untranslated or coding regions. Generally, a higher percentage of sequence identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective.

### C.3. Chimeroplasts

The SDFs of the invention, such as those described by Reference, Sequence, Protein Group, and Protein Group Matrix tables, can also be used to construct chimeroplasts that can be introduced into a cell to produce at least one specific nucleotide change in a sequence corresponding to the SDF of the invention. A chimeroplast is an oligonucleotide comprising

DNA and/or RNA that specifically hybridizes to a target region in a manner which creates a mismatched base-pair. This mismatched base-pair signals the cell's repair enzyme machinery which acts on the mismatched region resulting in the replacement, insertion or deletion of designated nucleotide(s). The altered sequence is then expressed by the cell's normal cellular mechanisms. Chimeroplasts can be designed to repair mutant genes, modify genes, introduce site-specific mutations, and/or act to interrupt or alter normal gene function (US Pat. Nos. 6,010,907 and 6,004,804; and PCT Pub. No. WO99/58723 and WO99/07865).

#### C.4. Sense Suppression

The SDFs of the reference, Sequence, Protein Group, and Protein Group Matrix tables of the present invention are also useful to modulate gene expression by sense suppression. Sense suppression represents another method of gene suppression by introducing at least one exogenous copy or fragment of the endogenous sequence to be suppressed.

Introduction of expression cassettes in which a nucleic acid is configured in the sense orientation with respect to the promoter into the chromosome of a plant or by a self-replicating virus has been shown to be an effective means by which to induce degradation of mRNAs of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al., *The Plant Cell* 2:279 (1990), and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184. Inhibition of expression may require some transcription of the introduced sequence.

For sense suppression, the introduced sequence generally will be substantially identical to the endogenous sequence intended to be inactivated. The minimal percentage of sequence identity will typically be greater than about 65%, but a higher percentage of sequence identity might exert a more effective reduction in the level of normal gene products. Sequence identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect would likely apply to any other proteins within a similar family of genes exhibiting homology or substantial homology to the suppressing sequence.

#### C.5. Transcriptional Silencing

The nucleic acid sequences of the invention, including the SDFs of the reference, Sequence, Protein Group, and Protein Group Matrix tables, and fragments thereof, contain

sequences that can be inserted into the genome of an organism resulting in transcriptional silencing. Such regulatory sequences need not be operatively linked to coding sequences to modulate transcription of a gene. Specifically, a promoter sequence without any other element of a gene can be introduced into a genome to transcriptionally silence an endogenous gene (see, for example, Vaucheret, H et al. (1998) *The Plant Journal* 16: 651-659). As another example, triple helices can be formed using oligonucleotides based on sequences from Reference, Sequence, Protein Group, and Protein Group Matrix tables, fragments thereof, and substantially similar sequence thereto. The oligonucleotide can be delivered to the host cell and can bind to the promoter in the genome to form a triple helix and prevent transcription. An oligonucleotide of interest is one that can bind to the promoter and block binding of a transcription factor to the promoter. In such a case, the oligonucleotide can be complementary to the sequences of the promoter that interact with transcription binding factors.

#### C.6. Other Methods to Inhibit Gene Expression

Yet another means of suppressing gene expression is to insert a polynucleotide into the gene of interest to disrupt transcription or translation of the gene.

Low frequency homologous recombination can be used to target a polynucleotide insert to a gene by flanking the polynucleotide insert with sequences that are substantially similar to the gene to be disrupted. Sequences from Reference, Sequence, Protein Group, and Protein Group Matrix tables, fragments thereof, and substantially similar sequence thereto can be used for homologous recombination.

In addition, random insertion of polynucleotides into a host cell genome can also be used to disrupt the gene of interest. Azpiroz-Leehan et al., *Trends in Genetics* 13:152 (1997). In this method, screening for clones from a library containing random insertions is preferred to identifying those that have polynucleotides inserted into the gene of interest. Such screening can be performed using probes and/or primers described above based on sequences from Reference, Sequence, Protein Group, and Protein Group Matrix tables, fragments thereof, and substantially similar sequence thereto. The screening can also be performed by selecting clones or R<sub>1</sub> plants having a desired phenotype.

I.D. Methods of Functional Analysis

The constructs described in the methods under I.C. above can be used to determine the function of the polypeptide encoded by the gene that is targeted by the constructs.

Down-regulating the transcription and translation of the targeted gene in the host cell or organisms, such as a plant, may produce phenotypic changes as compared to a wild-type cell or organism. In addition, *in vitro* assays can be used to determine if any biological activity, such as calcium flux, DNA transcription, nucleotide incorporation, etc., are being modulated by the down-regulation of the targeted gene.

Coordinated regulation of sets of genes, e.g., those contributing to a desired polygenic trait, is sometimes necessary to obtain a desired phenotype. SDFs of the invention representing transcription activation and DNA binding domains can be assembled into hybrid transcriptional activators. These hybrid transcriptional activators can be used with their corresponding DNA elements (i.e., those bound by the DNA-binding SDFs) to effect coordinated expression of desired genes (J.J. Schwarz et al., *Mol. Cell. Biol.* 12:266 (1992), A. Martinez et al., *Mol. Gen. Genet.* 261:546 (1999)).

The SDFs of the invention can also be used in the two-hybrid genetic systems to identify networks of protein-protein interactions (L. McAlister-Henn et al., *Methods* 19:330 (1999), J.C. Hu et al., *Methods* 20:80 (2000), M. Golovkin et al., *J. Biol. Chem.* 274:36428 (1999), K. Ichimura et al., *Biochem. Biophys. Res. Comm.* 253:532 (1998)). The SDFs of the invention can also be used in various expression display methods to identify important protein-DNA interactions (e.g. B. Luo et al., *J. Mol. Biol.* 266:479 (1997)).

I.E. Promoters

The SDFs of the invention are also useful as structural or regulatory sequences in a construct for modulating the expression of the corresponding gene in a plant or other organism, e.g. a symbiotic bacterium. For example, promoter sequences associated to SDFs of the reference, Sequence, Protein Group, and Protein Group Matrix tables of the present invention can be useful in directing expression of coding sequences either as constitutive promoters or to direct expression in particular cell types, tissues, or organs or in response to environmental stimuli.

With respect to the SDFs of the present invention a promoter is likely to be a relatively small portion of a genomic DNA (gDNA) sequence located in the first 2000 nucleotides upstream from an initial exon identified in a gDNA sequence or initial "ATG" or methionine codon or translational start site in a corresponding cDNA sequence. Such promoters are more likely to be found in the first 1000 nucleotides upstream of an initial ATG or methionine codon or translational start site of a cDNA sequence corresponding to a gDNA sequence. In particular, the promoter is usually located upstream of the transcription start site. The fragments of a particular gDNA sequence that function as elements of a promoter in a plant cell will preferably be found to hybridize to gDNA sequences presented and described in the Reference table at medium or high stringency, relevant to the length of the probe and its base composition.

Promoters are generally modular in nature. Promoters can consist of a basal promoter that functions as a site for assembly of a transcription complex comprising an RNA polymerase, for example RNA polymerase II. A typical transcription complex will include additional factors such as TF<sub>II</sub>B, TF<sub>II</sub>D, and TF<sub>II</sub>E. Of these, TF<sub>II</sub>D appears to be the only one to bind DNA directly. The promoter might also contain one or more enhancers and/or suppressors that function as binding sites for additional transcription factors that have the function of modulating the level of transcription with respect to tissue specificity and of transcriptional responses to particular environmental or nutritional factors, and the like.

Short DNA sequences representing binding sites for proteins can be separated from each other by intervening sequences of varying length. For example, within a particular functional module, protein binding sites may be constituted by regions of 5 to 60, preferably 10 to 30, more preferably 10 to 20 nucleotides. Within such binding sites, there are typically 2 to 6 nucleotides that specifically contact amino acids of the nucleic acid binding protein. The protein binding sites are usually separated from each other by 10 to several hundred nucleotides, typically by 15 to 150 nucleotides, often by 20 to 50 nucleotides. DNA binding sites in promoter elements often display dyad symmetry in their sequence. Often elements binding several different proteins, and/or a plurality of sites that bind the same protein, will be combined in a region of 50 to 1,000 basepairs.

Elements that have transcription regulatory function can be isolated from their corresponding endogenous gene, or the desired sequence can be synthesized, and recombined in constructs to direct expression of a coding region of a gene in a desired tissue-specific, temporal-

specific or other desired manner of inducibility or suppression. When hybridizations are performed to identify or isolate elements of a promoter by hybridization to the long sequences presented in the Reference tables, conditions are adjusted to account for the above-described nature of promoters. For example short probes, constituting the element sought, are preferably used under low temperature and/or high salt conditions. When long probes, which might include several promoter elements are used, low to medium stringency conditions are preferred when hybridizing to promoters across species.

If a nucleotide sequence of an SDF, or part of the SDF, functions as a promoter or fragment of a promoter, then nucleotide substitutions, insertions or deletions that do not substantially affect the binding of relevant DNA binding proteins would be considered equivalent to the exemplified nucleotide sequence. It is envisioned that there are instances where it is desirable to decrease the binding of relevant DNA binding proteins to silence or down-regulate a promoter, or conversely to increase the binding of relevant DNA binding proteins to enhance or up-regulate a promoter and vice versa. In such instances, polynucleotides representing changes to the nucleotide sequence of the DNA-protein contact region by insertion of additional nucleotides; changes to identity of relevant nucleotides, including use of chemically-modified bases, or deletion of one or more nucleotides are considered encompassed by the present invention. In addition, fragments of the promoter sequences described by Reference tables and variants thereof can be fused with other promoters or fragments to facilitate transcription and/or transcription in specific type of cells or under specific conditions.

Promoter function can be assayed by methods known in the art, preferably by measuring activity of a reporter gene operatively linked to the sequence being tested for promoter function. Examples of reporter genes include those encoding luciferase, green fluorescent protein, GUS, neo, cat and bar.

#### I.F. UTRs and Junctions

Polynucleotides comprising untranslated (UTR) sequences and intron/exon junctions are also within the scope of the invention. UTR sequences include introns and 5' or 3' untranslated regions ( 5' UTRs or 3' UTRs). Fragments of the sequences shown in the Reference and Sequence tables can comprise UTRs and intron/exon junctions.

These fragments of SDFs, especially UTRs, can have regulatory functions related to, for example, translation rate and mRNA stability. Thus, these fragments of SDFs can be isolated for use as elements of gene constructs for regulated production of polynucleotides encoding desired polypeptides.

Introns of genomic DNA segments might also have regulatory functions. Sometimes regulatory elements, especially transcription enhancer or suppressor elements, are found within introns. Also, elements related to stability of heteronuclear RNA and efficiency of splicing and of transport to the cytoplasm for translation can be found in intron elements. Thus, these segments can also find use as elements of expression vectors intended for use to transform plants.

Just as with promoters UTR sequences and intron/exon junctions can vary from those shown in the Reference and Sequence tables. Such changes from those sequences preferably will not affect the regulatory activity of the UTRs or intron/exon junction sequences on expression, transcription, or translation unless selected to do so. However, in some instances, down- or up-regulation of such activity may be desired to modulate traits or phenotypic or *in vitro* activity.

#### I.G. Coding Sequences

Isolated polynucleotides of the invention can include coding sequences that encode polypeptides comprising an amino acid sequence encoded by sequences described in the Reference and Sequence tables or an amino acid sequence presented in the Reference, Sequence, Protein Group, and Protein Group Matrix tables.

A nucleotide sequence encodes a polypeptide if a cell (or a cell free *in vitro* system) expressing that nucleotide sequence produces a polypeptide having the recited amino acid sequence when the nucleotide sequence is transcribed and the primary transcript is subsequently processed and translated by a host cell (or a cell free *in vitro* system) harboring the nucleic acid. Thus, an isolated nucleic acid that encodes a particular amino acid sequence can be a genomic sequence comprising exons and introns or a cDNA sequence that represents the product of splicing thereof. An isolated nucleic acid encoding an amino acid sequence also encompasses

heteronuclear RNA, which contains sequences that are spliced out during expression, and mRNA, which lacks those sequences.

Coding sequences can be constructed using chemical synthesis techniques or by isolating coding sequences or by modifying such synthesized or isolated coding sequences as described above.

In addition to coding sequences encoding the polypeptide sequences of the reference, Sequence, Protein Group, and Protein Group Matrix tables, which are native to corn, *Arabidopsis*, soybean, rice, wheat, and other plants, the isolated polynucleotides can be polynucleotides that encode variants, fragments, and fusions of those native proteins. Such polypeptides are described below in part II.

In variant polynucleotides generally, the number of substitutions, deletions or insertions is preferably less than 20%, more preferably less than 15%; even more preferably less than 10%, 5%, 3% or 1% of the number of nucleotides comprising a particularly exemplified sequence. It is generally expected that non-degenerate nucleotide sequence changes that result in 1 to 10, more preferably 1 to 5 and most preferably 1 to 3 amino acid insertions, deletions or substitutions will not greatly affect the function of an encoded polypeptide. The most preferred embodiments are those wherein 1 to 20, preferably 1 to 10, most preferably 1 to 5 nucleotides are added to, or deleted from and/or substituted in the sequences specifically disclosed in the Reference and Sequence tables or polynucleotides that encode polypeptides of the Protein Group, and Protein Group Matrix tables or fragments thereof.

Insertions or deletions in polynucleotides intended to be used for encoding a polypeptide preferably preserve the reading frame. This consideration is not so important in instances when the polynucleotide is intended to be used as a hybridization probe.

## II. Polypeptides and Proteins

### IIA. Native polypeptides and proteins

Polypeptides within the scope of the invention include both native proteins as well as variants, fragments, and fusions thereof. Polypeptides of the invention are those encoded by any of the six reading frames of sequences shown in the Reference and Sequence tables, preferably encoded by the three frames reading in the 5' to 3' direction of the sequences as shown.

Native polypeptides include the proteins encoded by the sequences shown in the Reference and Sequence tables. Such native polypeptides include those encoded by allelic variants.

Polypeptide and protein variants will exhibit at least 75% sequence identity to those native polypeptides of the Reference and Sequence tables. More preferably, the polypeptide variants will exhibit at least 85% sequence identity; even more preferably, at least 90% sequence identity; more preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity. Fragments of polypeptide or fragments of polypeptides will exhibit similar percentages of sequence identity to the relevant fragments of the native polypeptide. Fusions will exhibit a similar percentage of sequence identity in that fragment of the fusion represented by the variant of the native peptide.

Polypeptide and protein variants of the invention will exhibit at least 75% sequence identity to those motifs or consensus sequences of the Protein Group and Protein Group Matrix tables. More preferably, the polypeptide variants will exhibit at least 85% sequence identity; even more preferably, at least 90% sequence identity; more preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity. Fragments of polypeptide or fragments of polypeptides will exhibit similar percentages of sequence identity to the relevant fragments of the native polypeptide that are indicated in the Protein Group table. Fusions will exhibit a similar percentage of sequence identity in that fragment of the fusion represented by the variant of the native peptide.

Furthermore, polypeptide variants will exhibit at least one of the functional properties of the native protein. Such properties include, without limitation, protein interaction, DNA interaction, biological activity, immunological activity, receptor binding, signal transduction, transcription activity, growth factor activity, secondary structure, three-dimensional structure, etc. As to properties related to *in vitro* or *in vivo* activities, the variants preferably exhibit at least 60% of the activity of the native protein; more preferably at least 70%, even more preferably at least 80%, 85%, 90% or 95% of at least one activity of the native protein.

One type of variant of native polypeptides comprises amino acid substitutions, deletions and/or insertions. Conservative substitutions are preferred to maintain the function or activity of the polypeptide.

Within the scope of percentage of sequence identity described above, a polypeptide of the invention may have additional individual amino acids or amino acid sequences inserted into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide.

#### A.1 Antibodies

Isolated polypeptides can be utilized to produce antibodies. Polypeptides of the invention can generally be used, for example, as antigens for raising antibodies by known techniques. The resulting antibodies are useful as reagents for determining the distribution of the antigen protein within the tissues of a plant or within a cell of a plant. The antibodies are also useful for examining the production level of proteins in various tissues, for example in a wild-type plant or following genetic manipulation of a plant, by methods such as Western blotting.

Antibodies of the present invention, both polyclonal and monoclonal, may be prepared by conventional methods. In general, the polypeptides of the invention are first used to immunize a suitable animal, such as a mouse, rat, rabbit, or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies as detection reagents. Immunization is generally performed by mixing or emulsifying the protein in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization.

Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating the blood at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000xg for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Monoclonal antibodies are prepared using the method of Kohler and Milstein, *Nature* 256: 495 (1975), or modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells can be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate, or well, coated with the protein antigen. B-cells producing membrane-bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected Mab-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

Other methods for sustaining antibody-producing B-cell clones, such as by EBV transformation, are known.

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly <sup>32</sup>P and <sup>125</sup>I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TNB) to a blue pigment, quantifiable with a spectrophotometer.

#### A.2 In Vitro Applications of Polypeptides

Some polypeptides of the invention will have enzymatic activities that are useful *in vitro*. For example, the soybean trypsin inhibitor (Kunitz) family is one of the numerous families of proteinase inhibitors. It comprises plant proteins which have inhibitory activity against serine proteinases from the trypsin and subtilisin families, thiol proteinases and aspartic proteinases. Thus, these peptides find *in vitro* use in protein purification protocols and perhaps in therapeutic settings requiring topical application of protease inhibitors.

Delta-aminolevulinic acid dehydratase (EC 4.2.1.24) (ALAD) catalyzes the second step in the biosynthesis of heme, the condensation of two molecules of 5-aminolevulinate to form porphobilinogen and is also involved in chlorophyll biosynthesis(Kaczor et al. (1994) Plant Physiol. 1-4: 1411-7; Smith (1988) Biochem. J. 249: 423-8; Schneider (1976) Z. naturforsch. [C] 31: 55-63). Thus, ALAD proteins can be used as catalysts in synthesis of heme derivatives. Enzymes of biosynthetic pathways generally can be used as catalysts for *in vitro* synthesis of the compounds representing products of the pathway.

Polypeptides encoded by SDFs of the invention can be engineered to provide purification reagents to identify and purify additional polypeptides that bind to them. This allows one to identify proteins that function as multimers or elucidate signal transduction or metabolic pathways. In the case of DNA binding proteins, the polypeptide can be used in a similar manner to identify the DNA determinants of specific binding (S. Pierrou et al., *Anal. Biochem.* 229:99 (1995), S. Chusacultanachai et al., *J. Biol. Chem.* 274:23591 (1999), Q. Lin et al., *J. Biol. Chem.* 272:27274 (1997)).

## II.B. POLYPEPTIDE VARIANTS, FRAGMENTS, AND FUSIONS

Generally, variants , fragments, or fusions of the polypeptides encoded by the maximum length sequence(MLS) can exhibit at least one of the activities of the identified domains and/or related polypeptides described in Sections (C) and (D) of The Reference tables corresponding to the MLS of interest.

### II.B .(1)      Variants

A type of variant of the native polypeptides comprises amino acid substitutions. Conservative substitutions, described above (see II.), are preferred to maintain the function or activity of the polypeptide. Such substitutions include conservation of charge, polarity, hydrophobicity, size, etc. For example, one or more amino acid residues within the sequence can be substituted with another amino acid of similar polarity that acts as a functional equivalent, for example providing a hydrogen bond in an enzymatic catalysis. Substitutes for an amino acid within an exemplified sequence are preferably made among the members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine,

isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Within the scope of percentage of sequence identity described above, a polypeptide of the invention may have additional individual amino acids or amino acid sequences inserted into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide. Amino acid substitutions may also be made in the sequences; conservative substitutions being preferred.

One preferred class of variants are those that comprise (1) the domain of an encoded polypeptide and/or (2) residues conserved between the encoded polypeptide and related polypeptides. For this class of variants, the encoded polypeptide sequence is changed by insertion, deletion, or substitution at positions flanking the domain and/or conserved residues.

Another class of variants includes those that comprise an encoded polypeptide sequence that is changed in the domain or conserved residues by a conservative substitution.

Yet another class of variants includes those that lack one of the *in vitro* activities, or structural features of the encoded polypeptides. One example is polypeptides or proteins produced from genes comprising dominant negative mutations. Such a variant may comprise an encoded polypeptide sequence with non-conservative changes in a particular domain or group of conserved residues.

#### II.A.(2) FRAGMENTS

Fragments of particular interest are those that comprise a domain identified for a polypeptide encoded by an MLS of the instant invention and variants thereof. Also, fragments that comprise at least one region of residues conserved between an MLS encoded polypeptide and its related polypeptides are of great interest. Fragments are sometimes useful as polypeptides corresponding to genes comprising dominant negative mutations are.

#### II.A.(3) FUSIONS

Of interest are chimeras comprising (1) a fragment of the MLS encoded polypeptide or variants thereof of interest and (2) a fragment of a polypeptide comprising the same domain. For example, an AP2 helix encoded by a MLS of the invention fused to second AP2 helix from ANT protein, which comprises two AP2 helices. The present invention also encompasses fusions of MLS encoded polypeptides, variants, or fragments thereof fused with related proteins or fragments thereof.

#### DEFINITION OF DOMAINS

The polypeptides of the invention may possess identifying domains as shown in The Reference tables. Specific domains within the MLS encoded polypeptides are indicated in The Reference tables. In addition, the domains within the MLS encoded polypeptide can be defined by the region that exhibits at least 70% sequence identity with the consensus sequences listed in the detailed description below of each of the domains.

The majority of the protein domain descriptions given in the protein domain table are obtained from Prosite, (<http://www.expasy.ch/prosite/>), and Pfam, (<http://pfam.wustl.edu/browse.shtml>). Examples of domain descriptions are listed in the Protein Domain table.

#### A. Activities of Polypeptides Comprising Signal Peptides

Polypeptides comprising signal peptides are a family of proteins that are typically targeted to (1) a particular organelle or intracellular compartment, (2) interact with a particular molecule or (3) for secretion outside of a host cell. Examples of polypeptides comprising signal peptides include, without limitation, secreted proteins, soluble proteins, receptors, proteins retained in the ER, etc.

These proteins comprising signal peptides are useful to modulate ligand-receptor interactions, cell-to-cell communication, signal transduction, intracellular communication, and activities and/or chemical cascades that take part in an organism outside or within of any particular cell.

One class of such proteins are soluble proteins which are transported out of the cell. These proteins can act as ligands that bind to receptor to trigger signal transduction or to permit communication between cells.

Another class is receptor proteins which also comprise a retention domain that lodges the receptor protein in the membrane when the cell transports the receptor to the surface of the cell. Like the soluble ligands, receptors can also modulate signal transduction and communication between cells.

In addition the signal peptide itself can serve as a ligand for some receptors. An example is the interaction of the ER targeting signal peptide with the signal recognition particle (SRP). Here, the SRP binds to the signal peptide, halting translation, and the resulting SRP complex then binds to docking proteins located on the surface of the ER, prompting transfer of the protein into the ER.

A description of signal peptide residue composition is described below in Subsection IV.C.1.

### III. Methods of Modulating Polypeptide Production

It is contemplated that polynucleotides of the invention can be incorporated into a host cell or in-vitro system to modulate polypeptide production. For instance, the SDFs prepared as described herein can be used to prepare expression cassettes useful in a number of techniques for suppressing or enhancing expression.

An example are polynucleotides comprising sequences to be transcribed, such as coding sequences, of the present invention can be inserted into nucleic acid constructs to modulate polypeptide production. Typically, such sequences to be transcribed are heterologous to at least one element of the nucleic acid construct to generate a chimeric gene or construct.

Another example of useful polynucleotides are nucleic acid molecules comprising regulatory sequences of the present invention. Chimeric genes or constructs can be generated

when the regulatory sequences of the invention linked to heterologous sequences in a vector construct. Within the scope of invention are such chimeric gene and/or constructs.

Also within the scope of the invention are nucleic acid molecules, whereof at least a part or fragment of these DNA molecules are presented in the Reference and Sequence tables or polynucleotide encoding polypeptides of the Protein Group or Protein Group Matrix tables of the present application, and wherein the coding sequence is under the control of its own promoter and/or its own regulatory elements. Such molecules are useful for transforming the genome of a host cell or an organism regenerated from said host cell for modulating polypeptide production.

Additionally, a vector capable of producing the oligonucleotide can be inserted into the host cell to deliver the oligonucleotide.

More detailed description of components to be included in vector constructs are described both above and below.

Whether the chimeric vectors or native nucleic acids are utilized, such polynucleotides can be incorporated into a host cell to modulate polypeptide production. Native genes and/or nucleic acid molecules can be effective when exogenous to the host cell.

Methods of modulating polypeptide expression includes, without limitation:

Suppression methods, such as

Antisense

Ribozymes

Co-suppression

Insertion of Sequences into the Gene to be Modulated

Regulatory Sequence Modulation.

as well as Methods for Enhancing Production, such as

Insertion of Exogenous Sequences; and

Regulatory Sequence Modulation.

### III.A. Suppression

Expression cassettes of the invention can be used to suppress expression of endogenous genes which comprise the SDF sequence. Inhibiting expression can be useful, for instance, to

tailor the ripening characteristics of a fruit (Oeller et al., *Science* 254:437 (1991)) or to influence seed size (WO98/07842) or to provoke cell ablation (Mariani et al., *Nature* 357: 384-387 (1992)).

As described above, a number of methods can be used to inhibit gene expression in plants, such as antisense, ribozyme, introduction of exogenous genes into a host cell, insertion of a polynucleotide sequence into the coding sequence and/or the promoter of the endogenous gene of interest, and the like.

#### III.A.1. Antisense

An expression cassette as described above can be transformed into host cell or plant to produce an antisense strand of RNA. For plant cells, antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805 (1988), and Hiatt et al., U.S. Patent No. 4,801,340.

#### III.A.2. Ribozymes

Similarly, ribozyme constructs can be transformed into a plant to cleave mRNA and down-regulate translation.

#### III.A.3. Co-Suppression

Another method of suppression is by introducing an exogenous copy of the gene to be suppressed. Introduction of expression cassettes in which a nucleic acid is configured in the sense orientation with respect to the promoter has been shown to prevent the accumulation of mRNA. A detailed description of this method is described above.

#### III.A.4. Insertion of Sequences into the Gene to be Modulated

Yet another means of suppressing gene expression is to insert a polynucleotide into the gene of interest to disrupt transcription or translation of the gene.

Homologous recombination could be used to target a polynucleotide insert to a gene using the Cre-Lox system (A.C. Vergunst et al., *Nucleic Acids Res.* 26:2729 (1998), A.C. Vergunst et al., *Plant Mol. Biol.* 38:393 (1998), H. Albert et al., *Plant J.* 7:649 (1995)).

In addition, random insertion of polynucleotides into a host cell genome can also be used to disrupt the gene of interest. Azpiroz-Leehan et al., *Trends in Genetics* 13:152 (1997). In this method, screening for clones from a library containing random insertions is preferred for identifying those that have polynucleotides inserted into the gene of interest. Such screening can be performed using probes and/or primers described above based on sequences from the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, fragments thereof, and substantially similar sequence thereto. The screening can also be performed by selecting clones or any transgenic plants having a desired phenotype.

#### III.A.5. Regulatory Sequence Modulation

The SDFs described in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, and fragments thereof are examples of nucleotides of the invention that contain regulatory sequences that can be used to suppress or inactivate transcription and/or translation from a gene of interest as discussed in I.C.5.

#### III.A.6. Genes Comprising Dominant-Negative Mutations

When suppression of production of the endogenous, native protein is desired it is often helpful to express a gene comprising a dominant negative mutation. Production of protein variants produced from genes comprising dominant negative mutations is a useful tool for research. Genes comprising dominant negative mutations can produce a variant polypeptide which is capable of competing with the native polypeptide, but which does not produce the native result. Consequently, over expression of genes comprising these mutations can titrate out an undesired activity of the native protein. For example, The product from a gene comprising a dominant negative mutation of a receptor can be used to constitutively activate or suppress a signal transduction cascade, allowing examination of the phenotype and thus the trait(s) controlled by that receptor and pathway. Alternatively, the protein arising from the gene comprising a dominant-negative mutation can be an inactive enzyme still capable of binding to the same substrate as the native protein and therefore competes with such native protein.

Products from genes comprising dominant-negative mutations can also act upon the native protein itself to prevent activity. For example, the native protein may be active only as a homo-multimer or as one subunit of a hetero-multimer. Incorporation of an inactive subunit into the multimer with native subunit(s) can inhibit activity.

Thus, gene function can be modulated in host cells of interest by insertion into these cells vector constructs comprising a gene comprising a dominant-negative mutation.

### III.B. Enhanced Expression

Enhanced expression of a gene of interest in a host cell can be accomplished by either (1) insertion of an exogenous gene; or (2) promoter modulation.

#### III.B.1. Insertion of an Exogenous Gene

Insertion of an expression construct encoding an exogenous gene can boost the number of gene copies expressed in a host cell.

Such expression constructs can comprise genes that either encode the native protein that is of interest or that encode a variant that exhibits enhanced activity as compared to the native protein. Such genes encoding proteins of interest can be constructed from the sequences from the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, fragments thereof, and substantially similar sequence thereto.

Such an exogenous gene can include either a constitutive promoter permitting expression in any cell in a host organism or a promoter that directs transcription only in particular cells or times during a host cell life cycle or in response to environmental stimuli.

#### III.B.2. Regulatory Sequence Modulation

The SDFs of the Reference and Sequence tables, and fragments thereof, contain regulatory sequences that can be used to enhance expression of a gene of interest. For example, some of these sequences contain useful enhancer elements. In some cases, duplication of enhancer elements or insertion of exogenous enhancer elements will increase expression of a desired gene from a particular promoter. As other examples, all II promoters require binding of a regulatory protein to be activated, while some promoters may need a protein that signals a promoter binding protein to

expose a polymerase binding site. In either case, over-production of such proteins can be used to enhance expression of a gene of interest by increasing the activation time of the promoter.

Such regulatory proteins are encoded by some of the sequences in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, fragments thereof, and substantially similar sequences thereto.

Coding sequences for these proteins can be constructed as described above.

#### IV. Gene Constructs and Vector Construction

To use isolated SDFs of the present invention or a combination of them or parts and/or mutants and/or fusions of said SDFs in the above techniques, recombinant DNA vectors which comprise said SDFs and are suitable for transformation of cells, such as plant cells, are usually prepared. The SDF construct can be made using standard recombinant DNA techniques (Sambrook et al. 1989) and can be introduced to the species of interest by *Agrobacterium*-mediated transformation or by other means of transformation (e.g., particle gun bombardment) as referenced below.

The vector backbone can be any of those typical in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs and PACs and vectors of the sort described by

- (a) BAC: Shizuya et al., Proc. Natl. Acad. Sci. USA 89: 8794-8797 (1992);  
Hamilton et al., Proc. Natl. Acad. Sci. USA 93: 9975-9979 (1996);
- (b) YAC: Burke et al., Science 236:806-812 (1987);
- (c) PAC: Sternberg N. et al., Proc Natl Acad Sci U S A. Jan;87(1):103-7 (1990);
- (d) Bacteria-Yeast Shuttle Vectors: Bradshaw et al., Nucl Acids Res 23: 4850-4856 (1995);
- (e) Lambda Phage Vectors: Replacement Vector, e.g., Frischauf et al., J. Mol Biol 170: 827-842 (1983); or Insertion vector, e.g., Huynh et al., In: Glover NM (ed) DNA Cloning: A practical Approach, Vol.1 Oxford: IRL Press (1985);
- (f) T-DNA gene fusion vectors :Walden et al., Mol Cell Biol 1: 175-194 (1990); and
- (g) Plasmid vectors: Sambrook et al., infra.

Typically, a vector will comprise the exogenous gene, which in its turn comprises an SDF of the present invention to be introduced into the genome of a host cell, and which gene may be an antisense construct, a ribozyme construct chimeraplant, or a coding sequence with any desired transcriptional and/or translational regulatory sequences, such as promoters, UTRs, and 3' end termination sequences. Vectors of the invention can also include origins of replication, scaffold attachment regions (SARs), markers, homologous sequences, introns, etc.

A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for over-expression, a plant promoter fragment may be employed that will direct transcription of the gene in all tissues of a regenerated plant. Alternatively, the plant promoter may direct transcription of an SDF of the invention in a specific tissue (tissue-specific promoters) or may be otherwise under more precise environmental control (inducible promoters).

If proper polypeptide production is desired, a polyadenylation region at the 3'-end of the coding region is typically included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences from genes or SDF or the invention may comprise a marker gene that confers a selectable phenotype on plant cells. The vector can include promoter and coding sequence, for instance. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or phosphinotricin.

#### IV.A. Coding Sequences

Generally, the sequence in the transformation vector and to be introduced into the genome of the host cell does not need to be absolutely identical to an SDF of the present invention. Also, it is not necessary for it to be full length, relative to either the primary transcription product or fully processed mRNA. Furthermore, the introduced sequence need not have the same intron or exon pattern as a native gene. Also, heterologous non-coding segments

can be incorporated into the coding sequence without changing the desired amino acid sequence of the polypeptide to be produced.

#### **IV.B. Promoters**

As explained above, introducing an exogenous SDF from the same species or an orthologous SDF from another species are useful to modulate the expression of a native gene corresponding to that SDF of interest. Such an SDF construct can be under the control of either a constitutive promoter or a highly regulated inducible promoter (e.g., a copper inducible promoter). The promoter of interest can initially be either endogenous or heterologous to the species in question. When re-introduced into the genome of said species, such promoter becomes exogenous to said species. Over-expression of an SDF transgene can lead to co-suppression of the homologous endogenous sequence thereby creating some alterations in the phenotypes of the transformed species as demonstrated by similar analysis of the chalcone synthase gene (Napoli et al., *Plant Cell* 2:279 (1990) and van der Krol et al., *Plant Cell* 2:291 (1990)). If an SDF is found to encode a protein with desirable characteristics, its over-production can be controlled so that its accumulation can be manipulated in an organ- or tissue-specific manner utilizing a promoter having such specificity.

Likewise, if the promoter of an SDF (or an SDF that includes a promoter) is found to be tissue-specific or developmentally regulated, such a promoter can be utilized to drive or facilitate the transcription of a specific gene of interest (e.g., seed storage protein or root-specific protein). Thus, the level of accumulation of a particular protein can be manipulated or its spatial localization in an organ- or tissue- specific manner can be altered.

#### **IV. C Signal Peptides**

SDFs of the present invention containing signal peptides are indicated in the Reference Sequence, the Protein Group and Protein Group Matrix tables. In some cases it may be desirable for the protein encoded by an introduced exogenous or orthologous SDF to be targeted (1) to a particular organelle intracellular compartment, (2) to interact with a particular molecule such as a membrane molecule or (3) for secretion outside of the cell harboring the introduced SDF. This will be accomplished using a signal peptide.

Signal peptides direct protein targeting, are involved in ligand-receptor interactions and act in cell to cell communication. Many proteins, especially soluble proteins, contain a signal peptide that targets the protein to one of several different intracellular compartments. In plants, these compartments include, but are not limited to, the endoplasmic reticulum (ER), mitochondria, plastids (such as chloroplasts), the vacuole, the Golgi apparatus, protein storage vesicles (PSV) and, in general, membranes. Some signal peptide sequences are conserved, such as the Asn-Pro-Ile-Arg amino acid motif found in the N-terminal propeptide signal that targets proteins to the vacuole (Marty (1999) *The Plant Cell* 11: 587-599). Other signal peptides do not have a consensus sequence *per se*, but are largely composed of hydrophobic amino acids, such as those signal peptides targeting proteins to the ER (Vitale and Denecke (1999) *The Plant Cell* 11: 615-628). Still others do not appear to contain either a consensus sequence or an identified common secondary sequence, for instance the chloroplast stromal targeting signal peptides (Keegstra and Cline (1999) *The Plant Cell* 11: 557-570). Furthermore, some targeting peptides are bipartite, directing proteins first to an organelle and then to a membrane within the organelle (e.g. within the thylakoid lumen of the chloroplast; see Keegstra and Cline (1999) *The Plant Cell* 11: 557-570). In addition to the diversity in sequence and secondary structure, placement of the signal peptide is also varied. Proteins destined for the vacuole, for example, have targeting signal peptides found at the N-terminus, at the C-terminus and at a surface location in mature, folded proteins. Signal peptides also serve as ligands for some receptors.

These characteristics of signal proteins can be used to more tightly control the phenotypic expression of introduced SDFs. In particular, associating the appropriate signal sequence with a specific SDF can allow sequestering of the protein in specific organelles (plastids, as an example), secretion outside of the cell, targeting interaction with particular receptors, etc. Hence, the inclusion of signal proteins in constructs involving the SDFs of the invention increases the range of manipulation of SDF phenotypic expression. The nucleotide sequence of the signal peptide can be isolated from characterized genes using common molecular biological techniques or can be synthesized *in vitro*.

In addition, the native signal peptide sequences, both amino acid and nucleotide, described in the Reference, Sequence, Protein Group or Protein Group Matrix tables can be used to modulate polypeptide transport. Further variants of the native signal peptides described in the

Reference, Sequence, Protein Group or Protein Group Matrix tables are contemplated. Insertions, deletions, or substitutions can be made. Such variants will retain at least one of the functions of the native signal peptide as well as exhibiting some degree of sequence identity to the native sequence.

Also, fragments of the signal peptides of the invention are useful and can be fused with other signal peptides of interest to modulate transport of a polypeptide.

#### V. Transformation Techniques

A wide range of techniques for inserting exogenous polynucleotides are known for a number of host cells, including, without limitation, bacterial, yeast, mammalian, insect and plant cells.

Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, e.g. Weising et al., *Ann. Rev. Genet.* 22:421 (1988); and Christou, *Euphytica*, v. 85, n.1-3:13-27, (1995).

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria (McCormac et al., *Mol. Biotechnol.* 8:199 (1997); Hamilton, *Gene* 200:107 (1997)); Salomon et al. *EMBO J.* 3:141 (1984); Herrera-Estrella et al. *EMBO J.* 2:987 (1983).

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. *EMBO J.* 3:2717 (1984). Electroporation techniques are described in Fromm et al. *Proc. Natl Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein et al. *Nature* 327:773 (1987). *Agrobacterium tumefaciens*-mediated

transformation techniques, including disarming and use of binary or co-integrate vectors, are well described in the scientific literature. See, for example Hamilton, *CM., Gene* 200:107 (1997); Müller et al. *Mol. Gen. Genet.* 207:171 (1987); Komari et al. *Plant J.* 10:165 (1996); Venkateswarlu et al. *Biotechnology* 9:1103 (1991) and Gleave, *AP., Plant Mol. Biol.* 20:1203 (1992); Graves and Goldman, *Plant Mol. Biol.* 7:34 (1986) and Gould et al., *Plant Physiology* 95:426 (1991).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype such as seedlessness. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture* in "Handbook of Plant Cell Culture," pp. 124-176, MacMillan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1988. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Kleé et al. *Ann. Rev. of Plant Phys.* 38:467 (1987). Regeneration of monocots (rice) is described by Hosoyama et al. (*Biosci. Biotechnol. Biochem.* 58:1500 (1994)) and by Ghosh et al. (*J. Biotechnol.* 32:1 (1994)). The nucleic acids of the invention can be used to confer desired traits on essentially any plant.

Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium*, *Arachis*, *Asparagus*, *Atropa*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*, *Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Linum*, *Lolium*, *Lupinus*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nicotiana*, *Olea*, *Oryza*, *Panicum*, *Pannisetum*, *Persea*, *Phaseolus*, *Pistacia*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*, *Theobromus*, *Trigonella*, *Triticum*, *Vicia*, *Vitis*, *Vigna*, and, *Zea*.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

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The particular sequences of SDFs identified are provided in the attached Reference and Sequence tables.

## IX. DEFINITIONS

The following terms are utilized throughout this application:

**Allelic variant:** An "allelic variant" is an alternative form of the same SDF, which resides at the same chromosomal locus in the organism. Allelic variations can occur in any portion of the gene sequence, including regulatory regions. Allelic variants can arise by normal genetic variation in a population. Allelic variants can also be produced by genetic engineering methods. An allelic variant can be one that is found in a naturally occurring plant, including a cultivar or ecotype. An allelic variant may or may not give rise to a phenotypic change, and may or may not be expressed. An allele can result in a detectable change in the phenotype of the trait represented by the locus. A phenotypically silent allele can give rise to a product.

**Alternatively spliced messages:** Within the context of the current invention, "alternatively spliced messages" refers to mature mRNAs originating from a single gene with variations in the number and/or identity of exons, introns and/or intron-exon junctions.

**Chimeric:** The term "chimeric" is used to describe genes, as defined supra, or constructs wherein at least two of the elements of the gene or construct, such as the promoter and the coding sequence and/or other regulatory sequences and/or filler sequences and/or complements thereof, are heterologous to each other.

**Constitutive Promoter:** Promoters referred to herein as "constitutive promoters" actively promote transcription under most, but not necessarily all, environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcript initiation region and the 1' or 2' promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes, such as the maize ubiquitin-1 promoter, known to those of skill.

**Coordinately Expressed:** The term "coordinately expressed," as used in the current invention, refers to genes that are expressed at the same or a similar time and/or stage and/or under the same or similar environmental conditions.

**Domain:** Domains are fingerprints or signatures that can be used to characterize protein families and/or parts of proteins. Such fingerprints or signatures can comprise conserved (1) primary sequence, (2) secondary structure, and/or (3) three-dimensional conformation.

Generally, each domain has been associated with either a family of proteins or motifs. Typically, these families and/or motifs have been correlated with specific *in-vitro* and/or *in-vivo* activities. A domain can be any length, including the entirety of the sequence of a protein. Detailed descriptions of the domains, associated families and motifs, and correlated activities of the polypeptides of the instant invention are described below. Usually, the polypeptides with designated domain(s) can exhibit at least one activity that is exhibited by any polypeptide that comprises the same domain(s).

**Endogenous:** The term "endogenous," within the context of the current invention refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell or organisms regenerated from said cell.

**Exogenous:** "Exogenous," as referred to within, is any polynucleotide, polypeptide or protein sequence, whether chimeric or not, that is initially or subsequently introduced into the genome of an individual host cell or the organism regenerated from said host cell by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation (of dicots - e.g. Salomon et al. *EMBO J.* 3:141 (1984); Herrera-Estrella et al. *EMBO J.* 2:987 (1983); of monocots, representative papers are those by Escudero et al., *Plant J.* 10:355 (1996), Ishida et al., *Nature Biotechnology* 14:745 (1996), May et al., *Bio/Technology* 13:486 (1995)), biolistic methods (Armaleo et al., *Current Genetics* 17:97 1990)), electroporation, *in planta* techniques, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T<sub>0</sub> for the primary transgenic plant and T<sub>1</sub> for the first generation. The term "exogenous" as used herein is

also intended to encompass inserting a naturally found element into a non-naturally found location.

**Filler sequence:** As used herein, “filler sequence” refers to any nucleotide sequence that is inserted into DNA construct to evoke a particular spacing between particular components such as a promoter and a coding region and may provide an additional attribute such as a restriction enzyme site.

**Gene:** The term “gene,” as used in the context of the current invention, encompasses all regulatory and coding sequence contiguously associated with a single hereditary unit with a genetic function (see SCHEMATIC 1). Genes can include non-coding sequences that modulate the genetic function that include, but are not limited to, those that specify polyadenylation, transcriptional regulation, DNA conformation, chromatin conformation, extent and position of base methylation and binding sites of proteins that control all of these. Genes comprised of “exons” (coding sequences), which may be interrupted by “introns” (non-coding sequences), encode proteins. A gene’s genetic function may require only RNA expression or protein production, or may only require binding of proteins and/or nucleic acids without associated expression. In certain cases, genes adjacent to one another may share sequence in such a way that one gene will overlap the other. A gene can be found within the genome of an organism, artificial chromosome, plasmid, vector, etc., or as a separate isolated entity.

**Gene Family:** “Gene family” is used in the current invention to describe a group of functionally related genes, each of which encodes a separate protein.

**Heterologous sequences:** “Heterologous sequences” are those that are not operatively linked or are not contiguous to each other in nature. For example, a promoter from corn is considered heterologous to an *Arabidopsis* coding region sequence. Also, a promoter from a gene encoding a growth factor from corn is considered heterologous to a sequence encoding the corn receptor for the growth factor. Regulatory element sequences, such as UTRs or 3’ end termination sequences that do not originate in nature from the same gene as the coding sequence originates from, are

considered heterologous to said coding sequence. Elements operatively linked in nature and - contiguous to each other are not heterologous to each other. On the other hand, these same elements remain operatively linked but become heterologous if other filler sequence is placed between them. Thus, the promoter and coding sequences of a corn gene expressing an amino acid transporter are not heterologous to each other, but the promoter and coding sequence of a corn gene operatively linked in a novel manner are heterologous.

**Homologous gene:** In the current invention, “homologous gene” refers to a gene that shares sequence similarity with the gene of interest. This similarity may be in only a fragment of the sequence and often represents a functional domain such as, examples including without limitation a DNA binding domain, a domain with tyrosine kinase activity, or the like. The functional activities of homologous genes are not necessarily the same.

**Inducible Promoter:** An “inducible promoter” in the context of the current invention refers to a promoter which is regulated under certain conditions, such as light, chemical concentration, protein concentration, conditions in an organism, cell, or organelle, etc. A typical example of an inducible promoter, which can be utilized with the polynucleotides of the present invention, is PARSK1, the promoter from the *Arabidopsis* gene encoding a serine-threonine kinase enzyme, and which promoter is induced by dehydration, abscissic acid and sodium chloride (Wang and Goodman, *Plant J.* 8:37 (1995)) Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light.

**Intergenic region:** “Intergenic region,” as used in the current invention, refers to nucleotide sequence occurring in the genome that separates adjacent genes.

**Mutant gene:** In the current invention, “mutant” refers to a heritable change in DNA sequence at a specific location. Mutants of the current invention may or may not have an associated identifiable function when the mutant gene is transcribed.

**Orthologous Gene:** In the current invention "orthologous gene" refers to a second gene that encodes a gene product that performs a similar function as the product of a first gene. The orthologous gene may also have a degree of sequence similarity to the first gene. The orthologous gene may encode a polypeptide that exhibits a degree of sequence similarity to a polypeptide corresponding to a first gene. The sequence similarity can be found within a functional domain or along the entire length of the coding sequence of the genes and/or their corresponding polypeptides.

**Percentage of sequence identity:** "Percentage of sequence identity," as used herein, is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the polynucleotide or amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Add. APL. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (USA)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. Given that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. The term "substantial sequence identity" between polynucleotide or polypeptide sequences refers to polynucleotide or polypeptide comprising a sequence that has at least 80% sequence identity, preferably at least 85%, more preferably at least 90% and most preferably at least 95%, even more preferably, at least 96%, 97%, 98% or 99% sequence identity compared to a reference sequence using the programs.

**Plant Promoter:** A “plant promoter” is a promoter capable of initiating transcription in plant cells and can drive or facilitate transcription of a fragment of the SDF of the instant invention or a coding sequence of the SDF of the instant invention. Such promoters need not be of plant origin. For example, promoters derived from plant viruses, such as the CaMV35S promoter or from *Agrobacterium tumefaciens* such as the T-DNA promoters, can be plant promoters. A typical example of a plant promoter of plant origin is the maize ubiquitin-1 (*ubi-1*)promoter known to those of skill.

**Promoter:** The term "promoter," as used herein, refers to a region of sequence determinants located upstream from the start of transcription of a gene and which are involved in recognition and binding of RNA polymerase and other proteins to initiate and modulate transcription. A basal promoter is the minimal sequence necessary for assembly of a transcription complex required for transcription initiation. Basal promoters frequently include a “TATA box” element usually located between 15 and 35 nucleotides upstream from the site of initiation of transcription. Basal promoters also sometimes include a “CCAAT box” element (typically a sequence CCAAT) and/or a GGGCG sequence, usually located between 40 and 200 nucleotides, preferably 60 to 120 nucleotides, upstream from the start site of transcription.

**Public sequence:** The term “public sequence,” as used in the context of the instant application, refers to any sequence that has been deposited in a publicly accessible database. This term encompasses both amino acid and nucleotide sequences. Such sequences are publicly accessible, for example, on the BLAST databases on the NCBI FTP web site (accessible at ncbi.nlm.gov/blast). The database at the NCBI GTP site utilizes “gi” numbers assigned by NCBI as a unique identifier for each sequence in the databases, thereby providing a non-redundant database for sequence from various databases, including GenBank, EMBL, DBBJ, (DNA Database of Japan) and PDB (Brookhaven Protein Data Bank).

**Regulatory Sequence:** The term “regulatory sequence,” as used in the current invention, refers to any nucleotide sequence that influences transcription or translation initiation and rate,

and stability and/or mobility of the transcript or polypeptide product. Regulatory sequences include, but are not limited to, promoters, promoter control elements, protein binding sequences, 5' and 3' UTRs, transcriptional start site, termination sequence, polyadenylation sequence, introns, certain sequences within a coding sequence, etc.

**Related Sequences:** “Related sequences” refer to either a polypeptide or a nucleotide sequence that exhibits some degree of sequence similarity with a sequence described by The Reference tables and The Sequence tables.

**Scaffold Attachment Region (SAR):** As used herein, “scaffold attachment region” is a DNA sequence that anchors chromatin to the nuclear matrix or scaffold to generate loop domains that can have either a transcriptionally active or inactive structure (Spiker and Thompson (1996) Plant Physiol. 110: 15-21).

**Sequence-determined DNA fragments (SDFs):** “Sequence-determined DNA fragments” as used in the current invention are isolated sequences of genes, fragments of genes, intergenic regions or contiguous DNA from plant genomic DNA or cDNA or RNA the sequence of which has been determined.

**Signal Peptide:** A “signal peptide” as used in the current invention is an amino acid sequence that targets the protein for secretion, for transport to an intracellular compartment or organelle or for incorporation into a membrane. Signal peptides are indicated in the tables and a more detailed description located below.

**Specific Promoter:** In the context of the current invention, “specific promoters” refers to a subset of inducible promoters that have a high preference for being induced in a specific tissue or cell and/or at a specific time during development of an organism. By “high preference” is meant at least 3-fold, preferably 5-fold, more preferably at least 10-fold still more preferably at least 20-fold, 50-fold or 100-fold increase in transcription in the desired tissue over the transcription in any other tissue. Typical examples of temporal and/or tissue specific promoters of plant origin

that can be used with the polynucleotides of the present invention, are: PTA29, a promoter which is capable of driving gene transcription specifically in tapetum and only during anther development (Koltonow et al., *Plant Cell* 2:1201 (1990); RCc2 and RCc3, promoters that direct root-specific gene transcription in rice (Xu et al., *Plant Mol. Biol.* 27:237 (1995); TobRB27, a root-specific promoter from tobacco (Yamamoto et al., *Plant Cell* 3:371 (1991)). Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues or organs, such as root, ovule, fruit, seeds, or flowers. Other suitable promoters include those from genes encoding storage proteins or the lipid body membrane protein, oleosin. A few root-specific promoters are noted above.

**Stringency:** "Stringency" as used herein is a function of probe length, probe composition (G + C content), and salt concentration, organic solvent concentration, and temperature of hybridization or wash conditions. Stringency is typically compared by the parameter  $T_m$ , which is the temperature at which 50% of the complementary molecules in the hybridization are hybridized, in terms of a temperature differential from  $T_m$ . High stringency conditions are those providing a condition of  $T_m - 5^\circ\text{C}$  to  $T_m - 10^\circ\text{C}$ . Medium or moderate stringency conditions are those providing  $T_m - 20^\circ\text{C}$  to  $T_m - 29^\circ\text{C}$ . Low stringency conditions are those providing a condition of  $T_m - 40^\circ\text{C}$  to  $T_m - 48^\circ\text{C}$ . The relationship of hybridization conditions to  $T_m$  (in  $^\circ\text{C}$ ) is expressed in the mathematical equation

$$T_m = 81.5 - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G+C) - (600/N) \quad (1)$$

where N is the length of the probe. This equation works well for probes 14 to 70 nucleotides in length that are identical to the target sequence. The equation below for  $T_m$  of DNA-DNA hybrids is useful for probes in the range of 50 to greater than 500 nucleotides, and for conditions that include an organic solvent (formamide).

$$T_m = 81.5 + 16.6 \log \{ [\text{Na}^+] / (1 + 0.7[\text{Na}^+]) \} + 0.41(\%G+C) - 500/L - 0.63(\%\text{formamide}) \quad (2)$$

where L is the length of the probe in the hybrid. (P. Tijessen, "Hybridization with Nucleic Acid Probes" in Laboratory Techniques in Biochemistry and Molecular Biology, P.C. vand der Vliet, ed., c. 1993 by Elsevier, Amsterdam.) The  $T_m$  of equation (2) is affected by the nature of the hybrid; for DNA-RNA hybrids  $T_m$  is 10-15°C higher than calculated, for RNA-RNA hybrids  $T_m$  is 20-25°C higher. Because the  $T_m$  decreases about 1 °C for each 1% decrease in homology when a long probe is used (Bonner et al., *J. Mol. Biol.* 81:123 (1973)), stringency conditions can be adjusted to favor detection of identical genes or related family members.

Equation (2) is derived assuming equilibrium and therefore, hybridizations according to the present invention are most preferably performed under conditions of probe excess and for sufficient time to achieve equilibrium. The time required to reach equilibrium can be shortened by inclusion of a hybridization accelerator such as dextran sulfate or another high volume polymer in the hybridization buffer.

Stringency can be controlled during the hybridization reaction or after hybridization has occurred by altering the salt and temperature conditions of the wash solutions used. The formulas shown above are equally valid when used to compute the stringency of a wash solution. Preferred wash solution stringencies lie within the ranges stated above; high stringency is 5-8°C below  $T_m$ , medium or moderate stringency is 26-29°C below  $T_m$  and low stringency is 45-48°C below  $T_m$ .

**Substantially free of:** A composition containing A is "substantially free of" B when at least 85% by weight of the total A+B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A+B in the composition, more preferably at least about 95% or even 99% by weight. For example, a plant gene or DNA sequence can be considered substantially free of other plant genes or DNA sequences.

**Translational start site:** In the context of the current invention, a "translational start site" is usually an ATG in the cDNA transcript, more usually the first ATG. A single cDNA, however, may have multiple translational start sites.

**Transcription start site:** “Transcription start site” is used in the current invention to describe the point at which transcription is initiated. This point is typically located about 25 nucleotides downstream from a TFIID binding site, such as a TATA box. Transcription can initiate at one or more sites within the gene, and a single gene may have multiple transcriptional start sites, some of which may be specific for transcription in a particular cell-type or tissue.

**Untranslated region (UTR):** A “UTR” is any contiguous series of nucleotide bases that is transcribed, but is not translated. These untranslated regions may be associated with particular functions such as increasing mRNA message stability. Examples of UTRs include, but are not limited to polyadenylation signals, terminations sequences, sequences located between the transcriptional start site and the first exon (5' UTR) and sequences located between the last exon and the end of the mRNA (3' UTR).

**Variant:** The term “variant” is used herein to denote a polypeptide or protein or polynucleotide molecule that differs from others of its kind in some way. For example, polypeptide and protein variants can consist of changes in amino acid sequence and/or charge and/or post-translational modifications (such as glycosylation, etc).

**X. EXAMPLES**

The invention is illustrated by way of the following examples. The invention is not limited by these examples as the scope of the invention is defined solely by the claims following.

**EXAMPLE 1: cDNA PREPARATION**

A number of the nucleotide sequences disclosed in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, herein as representative of the SDFs of the invention can be obtained by sequencing genomic DNA (gDNA) and/or cDNA from corn plants grown from HYBRID SEED # 35A19, purchased from Pioneer Hi-Bred International, Inc., Supply Management, P.O. Box 256, Johnston, Iowa 50131-0256.

A number of the nucleotide sequences disclosed in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables, herein as representative of the SDFs of the invention can also be obtained by sequencing genomic DNA from *Arabidopsis thaliana*, Wassilewskija ecotype or by sequencing cDNA obtained from mRNA from such plants as described below. This is a true breeding strain. Seeds of the plant are available from the Arabidopsis Biological Resource Center at the Ohio State University, under the accession number CS2360. Seeds of this plant were deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection, Manassas, VA on August 31, 1999, and were assigned ATCC No. PTA-595.

Other methods for cloning full-length cDNA are described, for example, by Seki et al., *Plant Journal* 15:707-720 (1998) "High-efficiency cloning of *Arabidopsis* full-length cDNA by biotinylated Cap trapper"; Maruyama et al., *Gene* 138:171 (1994) "Oligo-capping a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides"; and WO 96/34981.

Tissues were, or each organ was, individually pulverized and frozen in liquid nitrogen. Next, the samples were homogenized in the presence of detergents and then centrifuged. The debris and nuclei were removed from the sample and more detergents were added to the sample. The sample was centrifuged and the debris was removed. Then the sample was applied to a 2M sucrose cushion to isolate polysomes. The RNA was isolated by treatment with detergents and

proteinase K followed by ethanol precipitation and centrifugation. The polysomal RNA from the different tissues was pooled according to the following mass ratios: 15/15/1 for male inflorescences, female inflorescences and root, respectively. The pooled material was then used for cDNA synthesis by the methods described below.

Starting material for cDNA synthesis for the exemplary corn cDNA clones with sequences presented in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables was poly(A)-containing polysomal mRNAs from inflorescences and root tissues of corn plants grown from HYBRID SEED # 35A19. Male inflorescences and female (pre-and post-fertilization) inflorescences were isolated at various stages of development. Selection for poly(A) containing polysomal RNA was done using oligo d(T) cellulose columns, as described by Cox and Goldberg, "Plant Molecular Biology: A Practical Approach", pp. 1-35, Shaw ed., c. 1988 by IRL, Oxford. The quality and the integrity of the polyA+ RNAs were evaluated.

Starting material for cDNA synthesis for the exemplary *Arabidopsis* cDNA clones with sequences presented in the Reference and Sequence tables or polynucleotides encoding polypeptides of the Protein Group or Protein Group Matrix tables was polysomal RNA isolated from the top-most inflorescence tissues of *Arabidopsis thaliana* Wassilewskija (Ws.) and from roots of *Arabidopsis thaliana* Landsberg erecta (L. er.), also obtained from the Arabidopsis Biological Resource Center. Nine parts inflorescence to every part root was used, as measured by wet mass. Tissue was pulverized and exposed to liquid nitrogen. Next, the sample was homogenized in the presence of detergents and then centrifuged. The debris and nuclei were removed from the sample and more detergents were added to the sample. The sample was centrifuged and the debris was removed and the sample was applied to a 2M sucrose cushion to isolate polysomal RNA. Cox et al., "Plant Molecular Biology: A Practical Approach", pp. 1-35, Shaw ed., c. 1988 by IRL, Oxford. The polysomal RNA was used for cDNA synthesis by the methods described below. Polysomal mRNA was then isolated as described above for corn cDNA. The quality of the RNA was assessed electrophoretically.

Following preparation of the mRNAs from various tissues as described above, selection of mRNA with intact 5' ends and specific attachment of an oligonucleotide tag to the 5' end of such mRNA was performed using either a chemical or enzymatic approach. Both techniques take

advantage of the presence of the "cap" structure, which characterizes the 5' end of most intact mRNAs and which comprises a guanosine generally methylated once, at the 7 position.

The chemical modification approach involves the optional elimination of the 2', 3'-cis diol of the 3' terminal ribose, the oxidation of the 2', 3'-cis diol of the ribose linked to the cap of the 5' ends of the mRNAs into a dialdehyde, and the coupling of the such obtained dialdehyde to a derivatized oligonucleotide tag. Further detail regarding the chemical approaches for obtaining mRNAs having intact 5' ends are disclosed in International Application No. WO96/34981 published November 7, 1996.

The enzymatic approach for ligating the oligonucleotide tag to the intact 5' ends of mRNAs involves the removal of the phosphate groups present on the 5' ends of uncapped incomplete mRNAs, the subsequent decapping of mRNAs having intact 5' ends and the ligation of the phosphate present at the 5' end of the decapped mRNA to an oligonucleotide tag. Further detail regarding the enzymatic approaches for obtaining mRNAs having intact 5' ends are disclosed in Dumas Milne Edwards J.B. (Doctoral Thesis of Paris VI University, Le clonage des ADNc complets: difficultés et perspectives nouvelles. Apports pour l'étude de la régulation de l'expression de la tryptophane hydroxylase de rat, 20 Dec. 1993), EP0 625572 and Kato *et al.*, *Gene* 150:243-250 (1994).

In both the chemical and the enzymatic approach, the oligonucleotide tag has a restriction enzyme site (e.g. an EcoRI site) therein to facilitate later cloning procedures. Following attachment of the oligonucleotide tag to the mRNA, the integrity of the mRNA is examined by performing a Northern blot using a probe complementary to the oligonucleotide tag.

For the mRNAs joined to oligonucleotide tags using either the chemical or the enzymatic method, first strand cDNA synthesis is performed using an oligo-dT primer with reverse transcriptase. This oligo-dT primer can contain an internal tag of at least 4 nucleotides, which can be different from one mRNA preparation to another. Methylated dCTP is used for cDNA first strand synthesis to protect the internal EcoRI sites from digestion during subsequent steps. The first strand cDNA is precipitated using isopropanol after removal of RNA by alkaline hydrolysis to eliminate residual primers.

Second strand cDNA synthesis is conducted using a DNA polymerase, such as Klenow fragment and a primer corresponding to the 5' end of the ligated oligonucleotide. The primer is

typically 20-25 bases in length. Methylated dCTP is used for second strand synthesis in order to protect internal EcoRI sites in the cDNA from digestion during the cloning process.

Following second strand synthesis, the full-length cDNAs are cloned into a phagemid vector, such as pBlueScript<sup>TM</sup> (Stratagene). The ends of the full-length cDNAs are blunted with T4 DNA polymerase (Biolabs) and the cDNA is digested with EcoRI. Since methylated dCTP is used during cDNA synthesis, the EcoRI site present in the tag is the only hemi-methylated site; hence the only site susceptible to EcoRI digestion. In some instances, to facilitate subcloning, an Hind III adapter is added to the 3' end of full-length cDNAs.

The full-length cDNAs are then size fractionated using either exclusion chromatography (AcA, Biosepra) or electrophoretic separation which yields 3 to 6 different fractions. The full-length cDNAs are then directionally cloned either into pBlueScript<sup>TM</sup> using either the EcoRI and SmaI restriction sites or, when the Hind III adapter is present in the full-length cDNAs, the EcoRI and Hind III restriction sites. The ligation mixture is transformed, preferably by electroporation, into bacteria, which are then propagated under appropriate antibiotic selection.

Clones containing the oligonucleotide tag attached to full-length cDNAs are selected as follows.

The plasmid cDNA libraries made as described above are purified (e.g. by a column available from Qiagen). A positive selection of the tagged clones is performed as follows. Briefly, in this selection procedure, the plasmid DNA is converted to single stranded DNA using phage F1 gene II endonuclease in combination with an exonuclease (Chang et al., *Gene* 127:95 (1993)) such as exonuclease III or T7 gene 6 exonuclease. The resulting single stranded DNA is then purified using paramagnetic beads as described by Fry et al., *Biotechniques* 13: 124 (1992). Here the single stranded DNA is hybridized with a biotinylated oligonucleotide having a sequence corresponding to the 3' end of the oligonucleotide tag. Preferably, the primer has a length of 20-25 bases. Clones including a sequence complementary to the biotinylated oligonucleotide are selected by incubation with streptavidin coated magnetic beads followed by magnetic capture. After capture of the positive clones, the plasmid DNA is released from the magnetic beads and converted into double stranded DNA using a DNA polymerase such as ThermoSequenase<sup>TM</sup> (obtained from Amersham Pharmacia Biotech). Alternatively, protocols such as the Gene Trapper<sup>TM</sup> kit (Gibco BRL) can be used. The double stranded DNA is then transformed, preferably by electroporation, into bacteria. The

percentage of positive clones having the 5' tag oligonucleotide is typically estimated to be between 90 and 98% from dot blot analysis.

Following transformation, the libraries are ordered in microtiter plates and sequenced. The *Arabidopsis* library was deposited at the American Type Culture Collection on January 7, 2000 as "*E-coli* liba 010600" under the accession number PTA-1161.

#### I. EXAMPLE 2: Southern hybridizations

The SDFs of the invention can be used in Southern hybridizations as described above. The following describes extraction of DNA from nuclei of plant cells, digestion of the nuclear DNA and separation by length, transfer of the separated fragments to membranes, preparation of probes for hybridization, hybridization and detection of the hybridized probe.

The procedures described herein can be used to isolate related polynucleotides or for diagnostic purposes. Moderate stringency hybridization conditions, as defined above, are described in the present example. These conditions result in detection of hybridization between sequences having at least 70% sequence identity. As described above, the hybridization and wash conditions can be changed to reflect the desired percentage of sequence identity between probe and target sequences that can be detected.

In the following procedure, a probe for hybridization is produced from two PCR reactions using two primers from genomic sequence of *Arabidopsis thaliana*. As described above, the particular template for generating the probe can be any desired template.

The first PCR product is assessed to validate the size of the primer to assure it is of the expected size. Then the product of the first PCR is used as a template, with the same pair of primers used in the first PCR, in a second PCR that produces a labeled product used as the probe.

Fragments detected by hybridization, or other bands of interest, can be isolated from gels used to separate genomic DNA fragments by known methods for further purification and/or characterization.

#### Buffers for nuclear DNA extraction

##### 1. 10X HB

	1000 ml	
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40 mM spermidine	10.2 g	Spermine (Sigma S-2876) and spermidine (Sigma S-2501)
10 mM spermine	3.5 g	Stabilize chromatin and the nuclear membrane
0.1 M EDTA (disodium)	37.2 g	EDTA inhibits nuclease
0.1 M Tris	12.1 g	Buffer
0.8 M KCl	59.6 g	Adjusts ionic strength for stability of nuclei

Adjust pH to 9.5 with 10 N NaOH. It appears that there is a nuclease present in leaves.  
Use of pH 9.5 appears to inactivate this nuclease.

2. 2 M sucrose (684 g per 1000 ml)

Heat about half the final volume of water to about 50°C. Add the sucrose slowly then bring the mixture to close to final volume; stir constantly until it has dissolved. Bring the solution to volume.

3. Sarkosyl solution (lyses nuclear membranes)

	<u>1000 ml</u>
N-lauroyl sarcosine (Sarkosyl)	20.0 g
0.1 M Tris	12.1 g
0.04 M EDTA (Disodium)	14.9 g

Adjust the pH to 9.5 after all the components are dissolved and bring up to the proper volume.

4. 20% Triton X-100  
80 ml Triton X-100  
320 ml 1xHB (w/o  $\beta$ -ME and PMSF)  
Prepare in advance; Triton takes some time to dissolve

A. PROCEDURE

1. Prepare 1X "H" buffer (keep ice-cold during use)

	<u>1000 ml</u>
10X HB	100 ml
2 M sucrose	250 ml a non-ionic osmoticum
Water	634 ml
Added just before use:	
100 mM PMSF*	10 ml a protease inhibitor; protects nuclear membrane proteins
$\beta$ -mercaptoethanol	1 ml inactivates nuclease by reducing disulfide bonds

\*100 mM PMSF

(phenyl methyl sulfonyl fluoride, Sigma P-7626)

(add 0.0875 g to 5 ml 100% ethanol)

2. Homogenize the tissue in a blender (use 300-400 ml of 1xHB per blender). Be sure that you use 5-10 ml of HB buffer per gram of tissue. Blenders generate heat so be sure to keep the homogenate cold. It is necessary to put the blenders in ice periodically.

3. Add the 20% Triton X-100 (25 ml per liter of homogenate) and gently stir on ice for 20 min. This lyses plastid, but not nuclear, membranes.
4. Filter the tissue suspension through several nylon filters into an ice-cold beaker. The first filtration is through a 250-micron membrane; the second is through an 85-micron membrane; the third is through a 50-micron membrane; and the fourth is through a 20-micron membrane. Use a large funnel to hold the filters. Filtration can be sped up by gently squeezing the liquid through the filters.
5. Centrifuge the filtrate at 1200 x g for 20 min. at 4°C to pellet the nuclei.
6. Discard the dark green supernatant. The pellet will have several layers to it. One is starch; it is white and gritty. The nuclei are gray and soft. In the early steps, there may be a dark green and somewhat viscous layer of chloroplasts.

Wash the pellets in about 25 ml cold H buffer (with Triton X-100) and resuspend by swirling gently and pipetting. After the pellets are resuspended.

Pellet the nuclei again at 1200 - 1300 x g. Discard the supernatant.

Repeat the wash 3-4 times until the supernatant has changed from a dark green to a pale green. This usually happens after 3 or 4 resuspensions. At this point, the pellet is typically grayish white and very slippery. The Triton X-100 in these repeated steps helps to destroy the chloroplasts and mitochondria that contaminate the prep.

Resuspend the nuclei for a final time in a total of 15 ml of H buffer and transfer the suspension to a sterile 125 ml Erlenmeyer flask.

7. Add 15 ml, dropwise, cold 2% Sarkosyl, 0.1 M Tris, 0.04 M EDTA solution (pH 9.5) while swirling gently. This lyses the nuclei. The solution will become very viscous.

8. Add 30 grams of CsCl and gently swirl at room temperature until the CsCl is in solution. The mixture will be gray, white and viscous.
9. Centrifuge the solution at 11,400 x g at 4°C for at least 30 min. The longer this spin is, the firmer the protein pellicle.
10. The result is typically a clear green supernatant over a white pellet, and (perhaps) under a protein pellicle. Carefully remove the solution under the protein pellicle and above the pellet. Determine the density of the solution by weighing 1 ml of solution and add CsCl if necessary to bring to 1.57 g/ml. The solution contains dissolved solids (sucrose etc) and the refractive index alone will not be an accurate guide to CsCl concentration.
11. Add 20 µl of 10 mg/ml EtBr per ml of solution.
12. Centrifuge at 184,000 x g for 16 to 20 hours in a fixed-angle rotor.
13. Remove the dark red supernatant that is at the top of the tube with a plastic transfer pipette and discard. Carefully remove the DNA band with another transfer pipette. The DNA band is usually visible in room light; otherwise, use a long wave UV light to locate the band.
14. Extract the ethidium bromide with isopropanol saturated with water and salt. Once the solution is clear, extract at least two more times to ensure that all of the EtBr is gone. Be very gentle, as it is very easy to shear the DNA at this step. This extraction may take a while because the DNA solution tends to be very viscous. If the solution is too viscous, dilute it with TE.
15. Dialyze the DNA for at least two days against several changes (at least three times) of TE (10 mM Tris, 1mM EDTA, pH 8) to remove the cesium chloride.

16. Remove the dialyzed DNA from the tubing. If the dialyzed DNA solution contains a lot of debris, centrifuge the DNA solution at least at 2500 x g for 10 min. and carefully transfer the clear supernatant to a new tube. Read the A260 concentration of the DNA.
17. Assess the quality of the DNA by agarose gel electrophoresis (1% agarose gel) of the DNA. Load 50 ng and 100 ng (based on the OD reading) and compare it with known and good quality DNA. Undigested lambda DNA and a lambda-HindIII-digested DNA are good molecular weight makers.

Protocol for Digestion of Genomic DNA

Protocol:

1. The relative amounts of DNA for different crop plants that provide approximately a balanced number of genome equivalent is given in Table 3. Note that due to the size of the wheat genome, wheat DNA will be underrepresented. Lambda DNA provides a useful control for complete digestion.
2. Precipitate the DNA by adding 3 volumes of 100% ethanol. Incubate at -20°C for at least two hours. Yeast DNA can be purchased and made up at the necessary concentration, therefore no precipitation is necessary for yeast DNA.
3. Centrifuge the solution at 11,400 x g for 20 min. Decant the ethanol carefully (be careful not to disturb the pellet). Be sure that the residual ethanol is completely removed either by vacuum desiccation or by carefully wiping the sides of the tubes with a clean tissue.
4. Resuspend the pellet in an appropriate volume of water. Be sure the pellet is fully resuspended before proceeding to the next step. This may take about 30 min.

5. Add the appropriate volume of 10X reaction buffer provided by the manufacturer of the restriction enzyme to the resuspended DNA followed by the appropriate volume of enzymes. Be sure to mix it properly by slowly swirling the tubes.
6. Set-up the lambda digestion-control for each DNA that you are digesting.
7. Incubate both the experimental and lambda digests overnight at 37°C. Spin down condensation in a microfuge before proceeding.
8. After digestion, add 2 µl of loading dye (typically 0.25% bromophenol blue, 0.25% xylene cyanol in 15% Ficoll or 30% glycerol) to the lambda-control digests and load in 1% TPE-agarose gel (TPE is 90 mM Tris-phosphate, 2 mM EDTA, pH 8). If the lambda DNA in the lambda control digests are completely digested, proceed with the precipitation of the genomic DNA in the digests.
9. Precipitate the digested DNA by adding 3 volumes of 100% ethanol and incubating in – 20°C for at least 2 hours (preferably overnight).

**EXCEPTION:** *Arabidopsis* and yeast DNA are digested in an appropriate volume; they don't have to be precipitated.

10. Resuspend the DNA in an appropriate volume of TE (e.g., 22 µl x 50 blots = 1100 µl) and an appropriate volume of 10X loading dye (e.g., 2.4 µl x 50 blots = 120 µl). Be careful in pipetting the loading dye - it is viscous. Be sure you are pipetting the correct volume.

Table 3

Some guide points in digesting genomic DNA.

Species	Genome Size	Size Relative to Arabidopsis	Genome Equivalent to 2 µg Arabidopsis DNA	Amount of DNA per blot
Arabidopsis	120 Mb	1X	1X	2 µg
Brassica	1,100 Mb	9.2X	0.54X	10 µg
Corn	2,800 Mb	23.3X	0.43X	20 µg
Cotton	2,300 Mb	19.2X	0.52X	20 µg
Oat	11,300 Mb	94X	0.11X	20 µg
Rice	400 Mb	3.3X	0.75X	5 µg
Soybean	1,100 Mb	9.2X	0.54X	10 µg
Sugarbeet	758 Mb	6.3X	0.8X	10 µg
Sweetclover	1,100 Mb	9.2X	0.54X	10 µg
Wheat	16,000 Mb	133X	0.08X	20 µg
Yeast	15 Mb	0.12X	1X	0.25 µg

#### Protocol for Southern Blot Analysis

The digested DNA samples are electrophoresed in 1% agarose gels in 1x TPE buffer. Low voltage; overnight separations are preferred. The gels are stained with EtBr and photographed.

1. For blotting the gels, first incubate the gel in 0.25 N HCl (with gentle shaking) for about 15 min.
2. Then briefly rinse with water. The DNA is denatured by 2 incubations. Incubate (with shaking) in 0.5 M NaOH in 1.5 M NaCl for 15 min.

3. The gel is then briefly rinsed in water and neutralized by incubating twice (with shaking) in 1.5 M Tris pH 7.5 in 1.5 M NaCl for 15 min.
4. A nylon membrane is prepared by soaking it in water for at least 5 min, then in 6X SSC for at least 15 min. before use. (20x SSC is 175.3 g NaCl, 88.2 g sodium citrate per liter, adjusted to pH 7.0.)
5. The nylon membrane is placed on top of the gel and all bubbles in between are removed. The DNA is blotted from the gel to the membrane using an absorbent medium, such as paper toweling and 6x SCC buffer. After the transfer, the membrane may be lightly brushed with a gloved hand to remove any agarose sticking to the surface.
6. The DNA is then fixed to the membrane by UV crosslinking and baking at 80°C. The membrane is stored at 4°C until use.

B. PROTOCOL FOR PCR AMPLIFICATION OF GENOMIC FRAGMENTS IN ARABIDOPSIS

Amplification procedures:

1. Mix the following in a 0.20 ml PCR tube or 96-well PCR plate:

Volume	Stock	Final Amount or Conc.
0.5 µl	~ 10 ng/µl genomic DNA <sup>1</sup>	5 ng
2.5 µl	<u>10X PCR buffer</u>	20 mM Tris, 50 mM KCl
0.75 µl	50 mM MgCl <sub>2</sub>	1.5 mM

<sup>1</sup> Arabidopsis DNA is used in the present experiment, but the procedure is a general one.

1 $\mu$ l	10 pmol/ $\mu$ l Primer 1 (Forward)	10 pmol
1 $\mu$ l	10 pmol/ $\mu$ l Primer 2 (Reverse)	10 pmol
0.5 $\mu$ l	5 mM dNTPs	0.1 mM
0.1 $\mu$ l	5 units/ $\mu$ l Platinum Taq™ (Life Technologies, Gaithersburg, MD) DNA Polymerase	1 units
(to 25 $\mu$ l)	Water	

2. The template DNA is amplified using a Perkin Elmer 9700 PCR machine:

1) 94°C for 10 min. followed by

2) 5 cycles:	3) 5 cycles:	4) 25 cycles:
94 °C - 30 sec	94 °C - 30 sec	94 °C - 30 sec
62 °C - 30 sec	58 °C - 30 sec	53 °C - 30 sec
72 °C - 3 min	72 °C - 3 min	72 °C - 3 min

5) 72°C for 7 min. Then the reactions are stopped by chilling to 4°C.

The procedure can be adapted to a multi-well format if necessary.

#### Quantification and Dilution of PCR Products:

1. The product of the PCR is analyzed by electrophoresis in a 1% agarose gel. A linearized plasmid DNA can be used as a quantification standard (usually at 50, 100, 200, and 400 ng). These will be used as references to approximate the amount of PCR products. HindIII-digested Lambda DNA is useful as a molecular weight marker. The gel can be run fairly quickly; e.g., at 100 volts. The standard gel is examined to determine that the size of the PCR products is consistent with the expected size and if there are significant extra bands or smearable products in the PCR reactions.
2. The amounts of PCR products can be estimated on the basis of the plasmid standard.
3. For the small number of reactions that produce extraneous bands, a small amount of DNA from bands with the correct size can be isolated by dipping a sterile 10- $\mu$ l tip into the band while viewing through a UV Transilluminator. The small amount of agarose gel (with the DNA fragment) is used in the labeling reaction.

### C. PROTOCOL FOR PCR-DIG-LABELING OF DNA

#### Solutions:

Reagents in PCR reactions (diluted PCR products, 10X PCR Buffer, 50 mM MgCl<sub>2</sub>, 5 U/ $\mu$ l Platinum Taq Polymerase, and the primers)

10X dNTP + DIG-11-dUTP [1:5]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.65 mM dTTP, 0.35 mM DIG-11-dUTP)

10X dNTP + DIG-11-dUTP [1:10]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.81 mM dTTP, 0.19 mM DIG-11-dUTP)

10X dNTP + DIG-11-dUTP [1:15]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.875 mM dTTP, 0.125 mM DIG-11-dUTP)

TE buffer (10 mM Tris, 1 mM EDTA, pH 8)

Maleate buffer: In 700 ml of deionized distilled water, dissolve 11.61 g maleic acid and 8.77 g NaCl. Add NaOH to adjust the pH to 7.5. Bring the volume to 1 L. Stir for 15 min. and sterilize.

10% blocking solution: In 80 ml deionized distilled water, dissolve 1.16g maleic acid. Next, add NaOH to adjust the pH to 7.5. Add 10 g of the blocking reagent powder (Boehringer Mannheim, Indianapolis, IN, Cat. no. 1096176). Heat to 60°C while stirring to dissolve the powder. Adjust the volume to 100 ml with water. Stir and sterilize.

1% blocking solution: Dilute the 10% stock to 1% using the maleate buffer.

Buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH9.5). Prepared from autoclaved solutions of 1M Tris pH 9.5, 5 M NaCl, and 1 M MgCl<sub>2</sub> in autoclaved distilled water.

Procedure:

1. PCR reactions are performed in 25  $\mu$ l volumes containing:

PCR buffer	1X
MgCl <sub>2</sub>	1.5 mM
10X dNTP + DIG-11-dUTP	1X (please see the note below)
Platinum Taq™ Polymerase	1 unit
10 pg probe DNA	
10 pmol primer 1	

Note:

	<u>Use for:</u>
10X dNTP + DIG-11-dUTP (1:5)	< 1 kb
10X dNTP + DiG-11-dUTP (1:10)	1 kb to 1.8 kb
10X dNTP + DIG-11-dUTP (1:15)	> 1.8 kb

2. The PCR reaction uses the following amplification cycles:

1) 94°C for 10 min.

2) 5 cycles:	3) 5 cycles:	4) 25 cycles:
95°C - 30 sec	95°C - 30 sec	95°C - 30 sec
61°C - 1 min	59°C - 1 min	51°C - 1 min
73°C - 5 min	75°C - 5 min	73°C - 5 min

5) 72°C for 8 min. The reactions are terminated by chilling to 4°C (hold).

3. The products are analyzed by electrophoresis- in a 1% agarose gel, comparing to an aliquot of the unlabelled probe starting material.

4. The amount of DIG-labeled probe is determined as follows:

Make serial dilutions of the diluted control DNA in dilution buffer (TE: 10 mM Tris and 1 mM EDTA, pH 8) as shown in the following table:

<u>DIG-labeled control</u>	<u>Stepwise Dilution</u>	<u>Final Conc. (Dilution Name)</u>
5 ng/ $\mu$ l	1 $\mu$ l in 49 $\mu$ l TE	100 pg/ $\mu$ l (A)
100 pg/ $\mu$ l (A)	25 $\mu$ l in 25 $\mu$ l TE	50 pg/ $\mu$ l (B)
50 pg/ $\mu$ l (B)	25 $\mu$ l in 25 $\mu$ l TE	25 pg/ $\mu$ l (C)
25 pg/ $\mu$ l (C)	20 $\mu$ l in 30 $\mu$ l TE	10 pg/ $\mu$ l (D)

- a. Serial dilutions of a DIG-labeled standard DNA ranging from 100 pg to 10 pg are spotted onto a positively charged nylon membrane, marking the membrane lightly with a pencil to identify each dilution.
- b. Serial dilutions (e.g., 1:50, 1:2500, 1:10,000) of the newly labeled DNA probe are spotted.
- c. The membrane is fixed by UV crosslinking.
- d. The membrane is wetted with a small amount of maleate buffer and then incubated in 1% blocking solution for 15 min at room temp.

- e. The labeled DNA is then detected using alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim, Indianapolis, IN, cat. no. 1093274) and an NBT substrate according to the manufacturer's instruction.
- f. Spot intensities of the control and experimental dilutions are then compared to estimate the concentration of the PCR-DIG-labeled probe.

#### D. PREHYBRIDIZATION AND HYBRIDIZATION OF SOUTHERN BLOTS

## Solutions:

100% Formamide purchased from Gibco

20X SSC (1X = 0.15 M NaCl, 0.015 M Na<sub>3</sub>citrate)

per L: 175 g NaCl

87.5 g Na<sub>3</sub>citrate 2H<sub>2</sub>O

20% Sarkosyl (N-lauroyl-sarcosine)

20% SDS (sodium dodecyl sulphate)

10% Blocking Reagent: In 80 ml deionized distilled water, dissolve 1.16 g maleic acid.

Next, add NaOH to adjust the pH to 7.5. Add 10 g of the blocking reagent powder.

Heat to 60°C while stirring to dissolve the powder. Adjust the volume to 100 ml

with water. Stir and sterilize.

#### Prehybridization Mix:

<u>Final Concentration</u>	<u>Components</u>	<u>Volume (per 100 ml)</u>	<u>Stock</u>
50%	Formamide	50 ml	100%
5X	SSC	25 ml	20X

0.1%	Sarkosyl	0.5 ml	20%
0.02%	SDS	0.1 ml	20%
2%	Blocking Reagent	20 ml	10%
	Water	4.4 ml	

General Procedures:

1. Place the blot in a heat-sealable plastic bag and add an appropriate volume of prehybridization solution ( $30 \text{ ml}/100\text{cm}^2$ ) at room temperature. Seal the bag with a heat sealer, avoiding bubbles as much as possible. Lay down the bags in a large plastic tray (one tray can accommodate at least 4–5 bags). Ensure that the bags are lying flat in the tray so that the prehybridization solution is evenly distributed throughout the bag.  
Incubate the blot for at least 2 hours with gentle agitation using a waver shaker.
2. Denature DIG-labeled DNA probe by incubating for 10 min. at  $98^\circ\text{C}$  using the PCR machine and immediately cool it to  $4^\circ\text{C}$ .
3. Add probe to prehybridization solution (25 ng/ml;  $30 \text{ ml} = 750 \text{ ng}$  total probe) and mix well but avoid foaming. Bubbles may lead to background.
4. Pour off the prehybridization solution from the hybridization bags and add new prehybridization and probe solution mixture to the bags containing the membrane.
5. Incubate with gentle agitation for at least 16 hours.
6. Proceed to medium stringency post-hybridization wash:

Three times for 20 min. each with gentle agitation using 1X SSC, 1% SDS at  $60^\circ\text{C}$ .

All wash solutions must be prewarmed to  $60^\circ\text{C}$ . Use about 100 ml of wash solution per membrane.

To avoid background keep the membranes fully submerged to avoid drying in spots; agitate sufficiently to avoid having membranes stick to one another.

7. After the wash, proceed to immunological detection and CSPD development.

E. PROCEDURE FOR IMMUNOLOGICAL DETECTION WITH CSPD

Solutions:

Buffer 1: Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; adjusted to pH 7.5 with NaOH)

Washing buffer: Maleic acid buffer with 0.3% (v/v) Tween 20.

Blocking stock solution 10% blocking reagent in buffer 1. Dissolve (10X concentration): blocking reagent powder (Boehringer Mannheim, Indianapolis, IN, cat. no. 1096176) by constantly stirring on a 65°C heating block or heat in a microwave, autoclave and store at 4°C.

Buffer 2

(1X blocking solution): Dilute the stock solution 1:10 in Buffer 1.

Detection buffer: 0.1 M Tris, 0.1 M NaCl, pH 9.5

Procedure:

1. After the post-hybridization wash the blots are briefly rinsed (1-5 min.) in the maleate washing buffer with gentle shaking.
2. Then the membranes are incubated for 30 min. in Buffer 2 with gentle shaking.

3. Anti-DIG-AP conjugate (Boehringer Mannheim, Indianapolis, IN, cat. no. 1093274) at 75 mU/ml (1:10,000) in Buffer 2 is used for detection. 75 ml of solution can be used for 3 blots.
4. The membrane is incubated for 30 min. in the antibody solution with gentle shaking.
5. The membrane are washed twice in washing buffer with gentle shaking. About 250 mls is used per wash for 3 blots.
6. The blots are equilibrated for 2–5 min in 60 ml detection buffer.
7. Dilute CSPD (1:200) in detection buffer. (This can be prepared ahead of time and stored in the dark at 4°C).

The following steps must be done individually. Bags (one for detection and one for exposure) are generally cut and ready before doing the following steps.

8. The blot is carefully removed from the detection buffer and excess liquid removed without drying the membrane. The blot is immediately placed in a bag and 1.5 ml of CSPD solution is added. The CSPD solution can be spread over the membrane. Bubbles present at the edge and on the surface of the blot are typically removed by gentle rubbing. The membrane is incubated for 5 min. in CSPD solution.
9. Excess liquid is removed and the membrane is blotted briefly (DNA side up) on Whatman 3MM paper. Do not let the membrane dry completely.
10. Seal the damp membrane in a hybridization bag and incubate for 10 min at 37°C to enhance the luminescent reaction.
11. Expose for 2 hours at room temperature to X-ray film. Multiple exposures can be taken. Luminescence continues for at least 24 hours and signal intensity increases during the first hours.

### **EXAMPLE 3: MICROARRAY EXPERIMENTS AND RESULTS**

#### **Example 3: MICROARRAY EXPERIMENTS AND RESULTS**

##### **1. Sample Tissue Preparation**

(a) Roots

Seeds of *Arabidopsis thaliana* (Ws) were sterilized in full strength bleach for less than 5 min., washed more than 3 times in sterile distilled deionized water and plated on MS agar plates. The plates were placed at 4°C for 3 nights and then placed vertically into a growth chamber having 16 hr light/8 hr dark cycles, 23 °C, 70% relative humidity and ~11,000 LUX. After 2 weeks, the roots were cut from the agar, flash frozen in liquid nitrogen and stored at -80°C.(EXPT REP: 108439 and 108434)

(b) Root Hairless mutants

Plants mutant at the *rhl* gene locus lack root hairs. This mutation is maintained as a heterozygote.

Seeds of *Arabidopsis thaliana* (Landsberg erecta) mutated at the *rhl* gene locus were sterilized using 30% bleach with 1 ul/ml 20% Triton -X 100 and then vernalized at 4°C for 3 days before being plated onto GM agar plates. Plates were placed in growth chamber with 16 hr light/8 hr. dark, 23°C, 14,500-15,900 LUX, and 70% relative humidity for germination and growth.

After 7 days, seedlings were inspected for root hairs using a dissecting microscope. Mutants were harvested and the cotyledons removed so that only root tissue remained. Tissue was then flash frozen in liquid nitrogen and stored at -80C. (EXPT REP: 108433)

*Arabidopsis thaliana* (Landsberg erecta) seedlings grown and prepared as above were used as controls. (EXPT REP: 108433)

Alternatively, seeds of *Arabidopsis thaliana* (Landsberg erecta), heterozygous for the *rhl1* (root hairless) mutation, were surface-sterilized in 30% bleach containing 0.1% Triton X-100 and further rinsed in sterile water. They were then vernalized at 4°C for 4 days before being plated onto MS agar plates. The plates were maintained in a growth chamber at 24°C with 16 hr light/8 hr dark for germination and growth. After 10 days, seedling roots that expressed the phenotype (i.e. lacking root hairs) were cut below the hypocotyl junction, frozen in liquid

nitrogen and stored at -80°C. Those seedlings with the normal root phenotype (heterozygous or wt) were collected as described for the mutant and used as controls.

(c) Rosette Leaves, Stems, and Siliques *Arabidopsis thaliana* (Ws) seed was vernalized at 4°C for 3 days before sowing in Metro-mix soil type 350. Flats were placed in a growth chamber having 16 hr light/8 hr dark, 80% relative humidity, 23°C and 13,000 LUX for germination and growth. After 3 weeks, rosette leaves, stems, and siliques (see EXPT REP: 108436, 108437 and 108438) were harvested, flash frozen in liquid nitrogen and stored at -80°C until use. After 4 weeks, siliques (<5mm, 5-10 mm and >10 mm) were harvested, flash frozen in liquid nitrogen and stored at -80°C until use. 5 week old whole plants (used as controls) were harvested, flash frozen in liquid nitrogen and kept at -80°C until RNA was isolated.

(d) Trichomes

*Arabidopsis thaliana* (Colombia glabrous) inflorescences were used as a control and CS8143 (hairy inflorescence ecotype) inflorescences, having increased trichomes, were used as the experimental sample.

Approximately 10 µl of each type of seed was sown on a flat of 350 soil (containing 0.03% marathon) and vernalized at 4°C for 3 days. Plants were then grown at room temperature under fluorescent lighting. Young inflorescences were collected at 30 days for the control plants and 37 days for the experimental plants. Each inflorescence was cut into one-half inch (1/2") pieces, flash frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

(e) Germination

*Arabidopsis thaliana* seeds (ecotype Ws) were sterilized in bleach and rinsed with sterile water. The seeds were placed in 100mm petri plates containing soaked autoclaved filter paper. Plates were foil-wrapped and left at 4°C for 3 nights to vernalize. After cold treatment, the foil was removed and plates were placed into a growth chamber having 16 hr light/8 hr dark cycles, 23 °C, 70% relative humidity and ~11,000 lux. Seeds were collected 1 d (EXPT REP: 108461),

2 d (EXPT REP: 108462), 3 d (EXPT REP: 108463) and 4 d (EXPT REP: 108464) later, flash frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

(f) Shoot Apical Meristem

*Arabidopsis thaliana* (Landsberg erecta) plants mutant at the *stm* gene locus lack shoot meristems, produce aerial rosettes, have a reduced number of flowers per inflorescence, as well as a reduced number of petals, stamens and carpels, and is female sterile. This mutation is maintained as a heterozygote.

Seeds of *Arabidopsis thaliana* (Landsberg erecta) mutated at the *stm* locus were sterilized using 30% bleach with 1 ul/ml 20% Triton -X100. The seeds were vernalized at 4°C for 3 days before being plated onto GM agar plates. Half were then put into a 22°C, 24 hr light growth chamber and half in a 24°C 16 hr light/8 hr dark growth chamber having 14,500-15,900 LUX, and 70% relative humidity for germination and growth.

After 7 days, seedlings were examined for leaf primordia using a dissecting microscope. Presence of leaf primordia indicated a wild type phenotype. Mutants were selected based on lack of leaf primordia. Mutants were then harvested and hypocotyls removed leaving only tissue in the shoot region. Tissue was then flash frozen in liquid nitrogen and stored at -80°C.

Control tissue was isolated from 5 day old Landsberg erecta seedlings grown in the same manner as above. Tissue from the shoot region was harvested in the same manner as the *stm* tissue, but only contained material from the 24°C, 16 hr light/8 hr dark long day cycle growth chamber. (EXPT REP: 108453)

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 8 days. Seedlings were carefully removed from the sand and the outer layers of leaf sheath removed. About 2 mm sections were cut and flash frozen in liquid nitrogen prior to storage at -80°C. The tissues above the shoot apices (~1 cm long) were cut, treated as above and used as control tissue.

(g) Abscisic acid (ABA)

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having grown 16 hr light/8 hr dark, 13,000 LUX, 70% humidity, and 20°C and watered twice a week with 1 L of 1X Hoagland's solution. Approximately 1,000 14 day old plants were spayed with 200-250 mls of 100 µM ABA in a 0.02% solution of the detergent Silwet L-77. Whole seedlings, including roots, were harvested within a 15 to 20 minute time period at 1 hr and 6 hr after treatment, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 100 µM ABA for treatment. Control plants were treated with water. After 6 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(h) Auxin Responsive

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 13,000 LUX, 70% humidity, 20°C and watered twice a week with 1 L of 1X Hoagland's solution (recipe recited in Feldmann et al., (1987) Mol. Gen. Genet. 208: 1-9 and described as complete nutrient solution). Approximately 1,000 14 day old plants were spayed with 200-250 mls of 100 µM NAA in a 0.02% solution of the detergent Silwet L-77. Aerial tissues (everything above the soil line) were harvested within a 15 to 20 minute time period 1 hr and 6 hrs after treatment, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX.

Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 100 µM NAA for treatment. Control plants were treated with water. After 6 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(i) Cytokinin

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 13,000 LUX, 70% humidity, 20°C temperature and watered twice a week with 1 L of 1X Hoagland's solution. Approximately 1,000 14 day old plants were spayed with 200-250 mls of 100 µM BA in a 0.02% solution of the detergent Silwet L-77. Aerial tissues (everything above the soil line) were harvested within a 15 to 20 minute time period 1 hr and 6 hrs after treatment, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 100 µM BA for treatment. Control plants were treated with water. After 6 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(j) Brassinosteroid Responsive

Two separate experiments were performed, one with epi-brassinolide and one with the brassinosteroid biosynthetic inhibitor brassinazole.

In the epi-brassinolide experiments, seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) and the brassinosteroid biosynthetic mutant *dwf4-1* were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 11,000 LUX, 70% humidity and 22°C temperature. Four week old plants were spayed with a 1 µM solution of epi-brassinolide and shoot parts (unopened floral primordia and shoot

apical meristems) harvested three hours later. Tissue was flash-frozen in liquid nitrogen and stored at -80°C.(EXPT REP 108480)

In the brassinazole experiments, seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) were grown as described above. Four week old plants were spayed with a 1 µM solution of brassinazole and shoot parts (unopened floral primordia and shoot apical meristems) harvested three hours later. Tissue was flash-frozen in liquid nitrogen and stored at -80°C.(EXPT REP 108481)

In addition to the spray experiments, tissue was prepared from two different mutants; (1) a *dwf4-1* knock out mutant (EXPT REP: 108478) and (2) a mutant overexpressing the *dwf4-1* gene (EXPT REP: 108479).

Seeds of wild-type *Arabidopsis thaliana* (ecotype Wassilewskija) and of the *dwf4-1* knock out and overexpressor mutants were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 11,000 LUX, 70% humidity and 22°C temperature. Tissue from shoot parts (unopened floral primordia and shoot apical meristems) was flash-frozen in liquid nitrogen and stored at -80°C.

Another experiment was completed with seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr. dark) conditions, 13,000 LUX light intensity, 70% humidity, 20°C temperature and watered twice a week with 1 L 1X Hoagland's solution(recipe recited in Feldmann et al., (1987) Mol. Gen. Genet. 208: 1-9 and described as complete nutrient solution). Approximately 1,000 14 day old plants were spayed with 200-250 mls of 0.1 µM Epi-Brassinolite in 0.02% solution of the detergent Silwet L-77. At 1 hr. and 6 hrs. after treatment aerial tissues were harvested within a 15 to 20 minute time period and flash-frozen in liquid nitrogen.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 0.1 µM epi-brassinolide for treatment. Control plants

were treated with distilled deionized water. After 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

**(k) Gibberillic Acid**

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 13,000 LUX, 70% humidity, 20°C and watered twice a week with 1 L of 1X Hoagland's solution. Approximately 1,000 14 day old plants were sprayed with 200-250 mls of 100 µM gibberillic acid in a 0.02% solution of the detergent Silwet L-77. At 1 hr. and 6 hrs. after treatment, aerial tissues (everything above the soil line) were harvested within a 15 to 20 minute time period, flash-frozen in liquid nitrogen and stored at -80°C.

Alternatively, seeds of *Arabidopsis thaliana* (ecotype Ws) were sown in Metro-mix soil type 350 and left at 4°C for 3 days to vernalize. They were then transferred to a growth chamber having 16 hr light/8 hr dark, 13,000 LUX, 80% humidity, 20°C temperature and watered every four days with 1.5 L water. 14 days after germination, plants were sprayed with 100 µM gibberillic acid or with water. Aerial tissues were harvested 1 hr (EXPT REP: 108484), 6 hrs (EXPT REP: 108485), 12 hrs (EXPT REP: 108486), and 24 hrs post-treatment, flash frozen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 100 µM gibberillic acid for treatment. Control plants were treated with water. After 1 hr, 6 hr and 12 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

**(l) Nitrogen: High to Low**

Wild type *Arabidopsis thaliana* seeds (ecotype Ws) were surface sterilized with 30% Clorox, 0.1% Triton X-100 for 5 minutes. Seeds were then rinsed with 4-5 exchanges of sterile

double distilled deionized water. Seeds were vernalized at 4°C for 2-4 days in darkness. After cold treatment, seeds were plated on modified 1X MS media (without NH<sub>4</sub>NO<sub>3</sub> or KNO<sub>3</sub>), 0.5% sucrose, 0.5g/L MES pH5.7, 1% phytagar and supplemented with KNO<sub>3</sub> to a final concentration of 60 mM (high nitrate modified 1X MS media). Plates were then grown for 7 days in a Percival growth chamber at 22°C with 16 hr. light/8 hr dark.

Germinated seedlings were then transferred to a sterile flask containing 50 mL of high nitrate modified 1X MS liquid media. Seedlings were grown with mild shaking for 3 additional days at 22°C in 16 hr. light/8 hr dark (in a Percival growth chamber) on the high nitrate modified 1X MS liquid media.

After three days of growth on high nitrate modified 1X MS liquid media, seedlings were transferred either to a new sterile flask containing 50 mL of high nitrate modified 1X MS liquid media or to low nitrate modified 1X MS liquid media (containing 20 !M KNO<sub>3</sub>). Seedlings were grown in these media conditions with mild shaking at 22°C in 16 hr light/ 8 hr dark for the appropriate time points and whole seedlings harvested for total RNA isolation via the Trizol method (LifeTech.). The time points used for the microarray experiments were 10 min. (EXPT REP: 108454) and 1 hour (EXPT REP: 108455) time points for both the high and low nitrate modified 1X MS media.

Alternatively, seeds that were surface sterilized in 30% bleach containing 0.1% Triton X-100 and further rinsed in sterile water, were planted on MS agar, (0.5% sucrose) plates containing 50 mM KNO<sub>3</sub> (potassium nitrate). The seedlings were grown under constant light (3500 LUX) at 22°C. After 12 days, seedlings were transferred to MS agar plates containing either 1mM KNO<sub>3</sub> or 50 mM KNO<sub>3</sub>. Seedlings transferred to agar plates containing 50 mM KNO<sub>3</sub> were treated as controls in the experiment. Seedlings transferred to plates with 1mM KNO<sub>3</sub> were rinsed thoroughly with sterile MS solution containing 1 mM KNO<sub>3</sub>. There were ten plates per transfer. Root tissue was collected and frozen in 15 mL Falcon tubes at various time points which included 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 9 hours, 12 hours, 16 hours, and 24 hours.

Maize 35A19 Pioneer hybrid seeds were sown on flats containing sand and grown in a Conviron growth chamber at 25°C, 16 hr light/8 hr dark, ~13,000 LUX and 80% relative humidity. Plants were watered every three days with double distilled deionized water. Germinated seedlings

are allowed to grow for 10 days and were watered with high nitrate modified 1X MS liquid media (see above). On day 11, young corn seedlings were removed from the sand (with their roots intact) and rinsed briefly in high nitrate modified 1X MS liquid media. The equivalent of half a flat of seedlings were then submerged (up to their roots) in a beaker containing either 500 mL of high or low nitrate modified 1X MS liquid media (see above for details).

At appropriate time points, seedlings were removed from their respective liquid media, the roots separated from the shoots and each tissue type flash frozen in liquid nitrogen and stored at -80°C. This was repeated for each time point. Total RNA was isolated using the Trizol method (see above) with root tissues only.

Corn root tissues isolated at the 4 hr and 16 hr time points were used for the microarray experiments. Both the high and low nitrate modified 1X MS media were used.

(m) Nitrogen: Low to High

*Arabidopsis thaliana* ecotype Ws seeds were sown on flats containing 4 L of a 1:2 mixture of Grace Zonolite vermiculite and soil. Flats were watered with 3 L of water and vernalized at 4°C for five days. Flats were placed in a Conviron growth chamber having 16 hr light/8 hr dark at 20°C, 80% humidity and 17,450 LUX. Flats were watered with approximately 1.5 L of water every four days. Mature, bolting plants (24 days after germination) were bottom treated with 2 L of either a control (100 mM mannitol pH 5.5) or an experimental (50 mM ammonium nitrate, pH 5.5) solution. Roots, leaves and siliques were harvested separately 30, 120 and 240 minutes after treatment, flash frozen in liquid nitrogen and stored at -80°C.

Hybrid maize seed (Pioneer hybrid 35A19) were aerated overnight in deionized water. Thirty seeds were plated in each flat, which contained 4 liters of Grace zonolite vermiculite. Two liters of water were bottom fed and flats were kept in a Conviron growth chamber with 16 hr light/8 hr dark at 20°C and 80% humidity. Flats were watered with 1 L of tap water every three days. Five day old seedlings were treated as described above with 2 L of either a control (100 mM mannitol pH 6.5) solution or 1 L of an experimental (50 mM ammonium nitrate, pH 6.8) solution. Fifteen shoots per time point per treatment were harvested 10, 90 and 180 minutes after treatment, flash frozen in liquid nitrogen and stored at -80°C.

Alternatively, seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were left at 4°C for 3 days to vernalize. They were then sown on vermiculite in a growth chamber having 16 hours light/8 hours dark, 12,000-14,000 LUX, 70% humidity, and 20°C. They were bottom-watered with tap water, twice weekly. Twenty-four days old plants were sprayed with either water (control) or 0.6% ammonium nitrate at 4 µL/cm<sup>2</sup> of tray surface. Total shoots and some primary roots were cleaned of vermiculite, flash-frozen in liquid nitrogen and stored at -80°C.

(n) Methyl Jasmonate

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr. dark, 13,000 LUX, 70% humidity, 20°C temperature and watered twice a week with 1 L of a 1X Hoagland's solution. Approximately 1,000 14 day old plants were spayed with 200-250 mls of 0.001% methyl jasmonate in a 0.02% solution of the detergent Silwet L-77. At 1 hr and 6 hrs after treatment, whole seedlings, including roots, were harvested within a 15 to 20 minute time period, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 0.001% methyl jasmonate for treatment. Control plants were treated with water. After 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(O) SALICYLIC ACID

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in trays and left at 4°C for 4 days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr. dark, 13,000 LUX, 70% humidity, 20°C temperature and watered twice a week with 1 L of a 1X Hoagland's solution. Approximately 1,000 14 day old plants were spayed with 200-250 mls of 5 mM salicylic acid (solubilized in 70% ethanol) in a 0.02% solution of the detergent Silwet L-77.

At 1 hr and 6 hrs after treatment, whole seedlings, including roots, were harvested within a 15 to 20 minute time period flash-frozen in liquid nitrogen and stored at -80°C.

Alternatively, seeds of wild-type *Arabidopsis thaliana* (ecotype Columbia) and mutant CS3726 were sown in soil type 200 mixed with osmocote fertilizer and Marathon insecticide and left at 4°C for 3 days to vernalize. Flats were incubated at room temperature with continuous light. Sixteen days post germination plants were sprayed with 2 mM SA, 0.02% SilwettL-77 or control solution (0.02% SilwettL-77). Aerial parts or flowers were harvested 1 hr (EXPT REP: 108471 and 108472), 4 hr (EXPT REP: 108469 and 108470), 6 hr (EXPT REP: 108440,) 24 hr (EXPT REP: 108443, 107953 and 107960) and 3 weeks (EXPT REP: 108475, 108476) post-treatment flash frozen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 2 mM SA for treatment. Control plants were treated with water. After 12 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

**(P) WOUNDING**

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 70% humidity and 20°C. After 14 days, the leaves were wounded with forceps. Aerial tissues were harvested 1 hour and 6 hours after wounding. Aerial tissues from unwounded plants served as controls. Tissues were flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were wounded (one leaf nicked by scissors) and placed in 1-liter beakers of water for treatment. Control plants were

treated not wounded. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(q) Drought stress

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in pots and left at 4°C for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 150,000-160,000 LUX, 20°C and 70% humidity. After 14 days, aerial tissues were cut and left to dry on 3MM Whatman paper in a petri-plate for 1 hour and 6 hours. Aerial tissues exposed for 1 hour and 6 hours to 3 MM Whatman paper wetted with 1X Hoagland's solution served as controls. Tissues were harvested, flash-frozen in liquid nitrogen and stored at -80°C.

Alternatively, *Arabidopsis thaliana* (Ws) seed was vernalized at 4°C for 3 days before sowing in Metromix soil type 350. Flats were placed in a growth chamber with 23°C, 16 hr light/8 hr. dark, 80% relative humidity, ~13,000 LUX for germination and growth. Plants were watered with 1-1.5 L of water every four days. Watering was stopped 16 days after germination for the treated samples, but continued for the control samples. Rosette leaves and stems (EXPT REP 108477, 108482 and 108483), flowers (see EXPT REP: 108473, 108474) and siliques were harvested 2 d, 3 d, 4 d, 5 d, 6 d and 7 d (EXPT REP: 108473) after watering was stopped. Tissue was flash frozen in liquid nitrogen and kept at -80 °C until RNA was isolated. Flowers and siliques were also harvested on day 8 from plants that had undergone a 7 d drought treatment followed by 1 day of watering (EXPT REP: 108474). Control plants (whole plants) were harvested after 5 weeks, flash frozen in liquid nitrogen and stored as above.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in empty 1-liter beakers at room temperature for treatment. Control plants were placed in water. After 1 hr, 6 hr, 12 hr and 24 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(R) OSMOTIC STRESS

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 20°C, and 70% humidity. After 14 days, the aerial tissues were cut and placed on 3 MM Whatman paper in a petri-plate wetted with 20% PEG (polyethylene glycol-M<sub>r</sub> 8,000) in 1X Hoagland's solution. Aerial tissues on 3 MM Whatman paper containing 1X Hoagland's solution alone served as the control. Aerial tissues were harvested at 1 hour and 6 hours after treatment, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 20% PEG (polyethylene glycol-M<sub>r</sub> 8,000) for treatment. Control plants were treated with water. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 150mM NaCl for treatment. Control plants were treated with water. After 1 hr, 6hr, and 24 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(S) HEAT SHOCK TREATMENT

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize before being transferred to a growth chamber with 16 hr light/8 hr dark, 12,000-14,000 LUX, 70% humidity and 20°C, fourteen day old plants were transferred to a 42°C growth chamber and aerial tissues were harvested 1 hr and 6 hr after transfer. Control plants were left at 20°C and aerial tissues were harvested. Tissues were flashfrozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers containing 42°C water for treatment. Control plants were treated with water at 25°C. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(T) COLD SHOCK TREATMENT

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 20°C and 70% humidity. Fourteen day old plants were transferred to a 4°C dark growth chamber and aerial tissues were harvested 1 hour and 6 hours later. Control plants were maintained at 20°C and covered with foil to avoid exposure to light. Tissues were flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers containing 4°C water for treatment. Control plants were treated with water at 25°C. After 1 hr and 6 hr aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(U) OXIDATIVE STRESS- HYDROGEN PEROXIDE TREATMENT

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize. Before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 20°C and 70% humidity. Fourteen day old plants were sprayed with 5 mM H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) in a 0.02% Silwett L-77 solution. Control plants were sprayed with a 0.02% Silwett L-77 solution. Aerial tissues were harvested 1 hour and 6 hours after spraying, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 5 mM H<sub>2</sub>O<sub>2</sub> for treatment. Control plants were treated with water. After 1 hr, 6 hr and 24 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(V) NITRIC OXIDE TREATMENT

Seeds of *Arabidopsis thaliana* (Wassilewskija) were sown in trays and left at 4°C for three days to vernalize before being transferred to a growth chamber having 16 hr light/8 hr dark, 12,000-14,000 LUX, 20°C and 70% humidity. Fourteen day old plants were sprayed with 5 mM sodium nitroprusside in a 0.02% Silwett L-77 solution. Control plants were sprayed with a 0.02% Silwett L-77 solution. Aerial tissues were harvested 1 hour and 6 hours after spraying, flash-frozen in liquid nitrogen and stored at -80°C.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 7 days. Seedlings were carefully removed from the sand and placed in 1-liter beakers with 5 mM nitroprusside for treatment. Control plants were treated with water. After 1 hr, 6 hr and 12 hr, aerial and root tissues were separated and flash frozen in liquid nitrogen prior to storage at -80°C.

(w) S4 Immature Buds, Inflorescence Meristem

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. Inflorescences containing immature floral buds [stages 1-12; Smyth et al., 1990] as well as the inflorescence meristem were harvested and flash frozen in liquid nitrogen.

(x) S5 Flowers (Opened)

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. Mature, unpollinated flowers [stages 12-14; Smyth et al. 1990] were harvested and flash frozen in liquid nitrogen.

(y) S6 Siliques (All Stages)

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. Siliques bearing developing seeds containing post fertilization through pre-heart stage [0-72 hours after fertilization (HAF)], heart- through early curled cotyledon stage [72-120 HAF] and late-curled cotyledon stage [>120 HAF] embryos were harvested separately and pooled prior to RNA isolation in a mass ratio of 1:1:1. The tissues were then flash frozen in liquid nitrogen. Description of the stages of *Arabidopsis* embryogenesis used were reviewed by Bowman (1994).

(z) ARABIDOPSIS ENDOSPERM

*mea/mea* Fruits 0-10 mm

Seeds of *Arabidopsis thaliana* heterozygous for the *fertilization-independent endosperm1 (fie1)* [Ohad et al., 1996; ecotype Landsberg *erecta (Ler)*] were sown in pots and left at 4°C for two to three days to vernalize. Kiyosue et al. (1999) subsequently determined that *fie1* was allelic to the gametophytic maternal effect mutant *medea* (Grossniklaus et al., 1998). Imbibed seeds were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 1-2 siliques (fruits) bearing developing seeds just prior to dessication [9 days after flowering (DAF)] were selected from each plant and were hand-dissected to identify wild-type, *mea/+* heterozygotes, and *mea/mea* homozygous mutant plants. At this stage, homozygous *mea/mea* plants produce short siliques that contain >70% aborted seed and can be distinguished from those produced by wild-type (100% viable seed) and *mea/+* heterozygous (50% viable seed) plants (Ohad et al., 1996; Grossniklaus et al., 1998; Kiyosue et al., 1999). Siliques 0-10 mm in length containing developing seeds 0-9 DAF produced by homozygous *mea/mea* plants were harvested and flash frozen in liquid nitrogen.

Pods 0-10 mm (Control Tissue for Sample 70)

Seeds of *Arabidopsis thaliana* heterozygous for the *fertilization-independent endosperm1 (fie1)* [Ohad et al., 1996; ecotype Landsberg *erecta (Ler)*] were sown in pots and left at 4°C for two to three days to vernalize. Kiyosue et al. (1999) subsequently determined that *fie1* was allelic to the gametophytic maternal effect mutant *medea* (Grossniklaus et al., 1998). Imbibed seeds were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 1-2 siliques (fruits) bearing developing seeds just prior to dessication [9 days after flowering (DAF)] were selected from each plant and were hand-dissected to identify wild-type, *mea/+* heterozygotes, and *mea/mea* homozygous mutant plants. At this stage, homozygous *mea/mea* plants produce short siliques that contain >70% aborted seed and can be distinguished from those produced by wild-type (100% viable seed) and *mea/+* heterozygous (50% viable seed) plants (Ohad et al., 1996; Grossniklaus et al., 1998; Kiyosue et al., 1999). Siliques 0-10 mm in length containing developing seeds 0-9 DAF produced by segregating wild-type plants

were opened and the seeds removed. The remaining tissues (pods minus seed) were harvested and flash frozen in liquid nitrogen.

(aa) ARABIDOPSIS SEEDS

Fruits (pod + seed) 0-5 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of Arabidopsis embryogenesis used in this determination were summarized by Bowman (1994). Siliques 0-5 mm in length containing post fertilization through pre-heart stage [0-72 hours after fertilization (HAF)] embryos were harvested and flash frozen in liquid nitrogen.

Fruits(pod + seed) 5-10 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of Arabidopsis embryogenesis used in this determination were summarized by Bowman (1994). Siliques 5-10 mm in length containing heart- through early upturned-U- stage [72-120 hours after fertilization (HAF)] embryos were harvested and flash frozen in liquid nitrogen.

Fruits(pod + seed) >10 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Siliques >10 mm in length containing green, late upturned-U- stage [>120 hours after fertilization (HAF)-9 days after flowering (DAF)] embryos were harvested and flash frozen in liquid nitrogen.

Green Pods 5-10 mm (Control Tissue for Samples 72-74)

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Siliques 5-10 mm in length containing developing seeds 72-120 hours after fertilization (HAF) were opened and the seeds removed. The remaining tissues (green pods minus seed) were harvested and flash frozen in liquid nitrogen.

Green Seeds from Fruits >10 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were

grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Siliques lengths were then determined and used as an approximate determinant for embryonic stage. Green siliques >10 mm in length containing developing seeds up to 9 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

Brown Seeds from Fruits >10 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis used in this determination were summarized by Bowman (1994). Siliques lengths were then determined and used as an approximate determinant for embryonic stage. Yellowing siliques >10 mm in length containing brown, dessicating seeds >11 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

Green/Brown Seeds from Fruits >10 mm

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature. 3-4 siliques (fruits) bearing developing seeds were selected from at least 3 plants and were hand-dissected to determine what developmental stage(s) were represented by the enclosed embryos. Description of the stages of *Arabidopsis* embryogenesis

used in this determination were summarized by Bowman (1994). Siliques lengths were then determined and used as an approximate determinant for embryonic stage. Green siliques >10 mm in length containing both green and brown seeds >9 days after flowering (DAF)] were opened and the seeds removed and harvested and flash frozen in liquid nitrogen.

Mature Seeds (24 hours after imbibition)

Mature dry seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown onto moistened filter paper and left at 4°C for two to three days to vernalize. Imbibed seeds were then transferred to a growth chamber [16 hr light: 8 hr dark conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature], the emerging seedlings harvested after 48 hours and flash frozen in liquid nitrogen.

Mature Seeds (Dry)

Seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 70% humidity, and 22°C temperature and taken to maturity. Mature dry seeds are collected, dried for one week at 28°C, and vernalized for one week at 4°C before used as a source of RNA.

OVULES

Seeds of *Arabidopsis thaliana* heterozygous for *pistillata* (*pi*) [ecotype Landsberg *erecta* (*Ler*)] were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light: 8 hr dark) conditions, 7000-8000 LUX light intensity, 76% humidity, and 24°C temperature.

Inflorescences were harvested from seedlings about 40 days old. The inflorescences were cut into small pieces and incubated in the following enzyme solution (pH 5) at room temperature for 0.5-1 hr.: 0.2% pectolyase Y-23, 0.04% pectinase, 5 mM MES, 3% Sucrose and MS salts (1900 mg/l KNO<sub>3</sub>, 1650 mg/l NH<sub>4</sub>NO<sub>3</sub>, 370 mg/l MgSO<sub>4</sub> • 7 H<sub>2</sub>O, 170 mg/l KH<sub>2</sub>PO<sub>4</sub>, 440 mg/l CaCl<sub>2</sub> •

2 H<sub>2</sub>O, 6.2 mg/l H<sub>2</sub>BO<sub>3</sub>, 15.6 mg/l MnSO<sub>4</sub> • 4 H<sub>2</sub>O, 8.6 mg/l ZnSO<sub>4</sub> • 7 H<sub>2</sub>O, 0.25 mg/l NaMoO<sub>4</sub> • 2 H<sub>2</sub>O, 0.025 mg/l CuCO<sub>4</sub> • 5 H<sub>2</sub>O, 0.025 mg/l CoCl<sub>2</sub> • 6 H<sub>2</sub>O, 0.83 mg/l KI, 27.8 mg/l FeSO<sub>4</sub> • 7 H<sub>2</sub>O, 37.3 mg/l Disodium EDTA, pH 5.8). At the end of the incubation the mixture of inflorescence material and enzyme solution was passed through a size 60 sieve and then through a sieve with a pore size of 125 µm. Ovules greater than 125 µm in diameter were collected, rinsed twice in B5 liquid medium (2500 mg/l KNO<sub>3</sub>, 250 mg/l MgSO<sub>4</sub> • 7 H<sub>2</sub>O, 150 mg/l NaH<sub>2</sub>PO<sub>4</sub> • H<sub>2</sub>O, 150 mg/l CaCl<sub>2</sub> • 2 H<sub>2</sub>O, 134 mg/l (NH<sub>4</sub>)<sub>2</sub>CaCl<sub>2</sub> • SO<sub>4</sub>, 3 mg/l H<sub>2</sub>BO<sub>3</sub>, 10 mg/l MnSO<sub>4</sub> • 4 H<sub>2</sub>O, 2 ZnSO<sub>4</sub> • 7 H<sub>2</sub>O, 0.25 mg/l NaMoO<sub>4</sub> • 2 H<sub>2</sub>O, 0.025 mg/l CuCO<sub>4</sub> • 5 H<sub>2</sub>O, 0.025 mg/l CoCl<sub>2</sub> • 6 H<sub>2</sub>O, 0.75 mg/l KI, 40 mg/l EDTA sodium ferric salt, 20 g/l sucrose, 10 mg/l Thiamine hydrochloride, 1 mg/l Pyridoxine hydrochloride, 1 mg/l Nicotinic acid, 100 mg/l myo-inositol, pH 5.5), rinsed once in deionized water and flash frozen in liquid nitrogen. The supernatant from the 125 µm sieving was passed through subsequent sieves of 50 µm and 32 µm. The tissue retained in the 32 µm sieve was collected and mRNA prepared for use as a control.

(bb) Herbicide treatment

*Arabidopsis thaliana* (Ws) seeds were sterilized for 5 min. with 30% bleach, 50 µl Triton in a total volume of 50 ml. Seeds were vernalized at 4°C for 3 days before being plated onto GM agar plates at a density of about 144 seeds per plate. Plates were incubated in a Percival growth chamber having 16 hr light/8 hr dark, 80% relative humidity, 22 °C and 11,000 LUX for 14 days.

Plates were sprayed (~0.5 mls/plate) with water, Finale (1.128 g/L), Glean (1.88 g/L), RoundUp (0.01 g/L) or Trimec (0.08 g/L). Tissue was collected and flash frozen in liquid nitrogen at the following time points: 0, 1, 2, 4 (EXPT REP: 107871 (Finale), 107881 (Glean), 107896 (Round-up) and 107886 (Trimec)), 8, 12(EXPT REP: 108467 (Finale), 108468 (Glean), 108465 (Round-up) and 108466, 107891 (Trimec)), and 24 hours. Frozen tissue was stored at -80°C prior to RNA isolation.

(cc) Ap2

Seeds of *Arabidopsis thaliana* (ecotype Landesberg erecta) and floral mutant *apetala2* (Jofuku et al., 1994, Plant Cell 6:1211-1225) were sown in pots and left at 4°C for two to three days to vernalize. They were then transferred to a growth chamber. Plants were grown under long-day (16 hr light, 8 hr dark) conditions 7000-8000 LUX light intensity, 70% humidity and 22 °C temperature. Inflorescences containing immature floral buds (stages 1-7; Bowman, 1994) as well as the inflorescence meristem were harvested and flashfrozen. Polysomal polyA+ RNA was isolated from tissue according to Cox and Goldberg, 1988).

(dd) Protein Degradation

*Arabidopsis thaliana* (ecotype Ws) wild-type and 13B12-1 (homozygous) mutant seed were sown in pots containing Metro-mix 350 soil and incubated at 4°C for four days. Vernalized seeds were germinated in the greenhouse (16 hr light/8 hr dark) over a 7 day period. Mutant seedlings were sprayed with 0.02% (active ingredient) Finale to confirm their transgenic standing. Plants were grown until the mutant phenotype (either multiple pistils in a single flower and/or multiple branching per node) was apparent. Young inflorescences immediately forming from the multiple-branched stems were cut and flash frozen in liquid nitrogen. Young inflorescences from wild-type plants grown in parallel and under identical conditions were collected as controls. All collected tissue was stored at -80°C until RNA isolation. (EXPT REP 108451)

(ee) Root tips

Seeds of *Arabidopsis thaliana* (ecotype Ws) were placed on MS plates and vernalized at 4°C for 3 days before being placed in a 25°C growth chamber having 16 hr light/8 hr dark, 70% relative humidity and about 3 W/m<sup>2</sup>. After 6 days, young seedlings were transferred to flasks containing B5 liquid medium, 1% sucrose and 0.05 mg/l indole-3-butyric acid. Flasks were incubated at room temperature with 100 rpm agitation. Media was replaced weekly. After three weeks, roots were harvested and incubated for 1 hr with 2% pectinase, 0.2% cellulase, pH 7 before straining through a #80 (Sigma) sieve. The root body material remaining on the sieve (used as the control) was flash frozen and stored at -80°C until use. The material that passed

through the #80 sieve was strained through a #200 (Sigma) sieve and the material remaining on the sieve (root tips) was flash frozen and stored at -80°C until use. Approximately 10 mg of root tips were collected from one flask of root culture.

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. Covered flats were watered every three days for 8 days. Seedlings were carefully removed from the sand and the root tips (~2 mm long) were removed and flash frozen in liquid nitrogen prior to storage at -80°C. The tissues above the root tips (~1 cm long) were cut, treated as above and used as control tissue.

(ff) rt1

The *rt1* allele is a variation of *rt1 rootless1* and is recessive. Plants displaying the *rt1* phenotype have few or no secondary roots.

Seed from plants segregating for *rt1* were sown on sand and placed in a growth chamber having 16 hr light/8 hr dark, 13,000 LUX, 70% humidity and 20°C temperature. Plants were watered every three days with tap water. Eleven (11) day old seedlings were carefully removed from the sand, keeping the roots intact. *rt1*-type seedlings were separated from their wild-type counterparts and the root tissue isolated. Root tissue from normal seedlings (control) and *rt1* mutants were flash frozen in liquid nitrogen and stored at -80°C until use.

(gg) Imbibed seed

Seeds of maize hybrid 35A (Pioneer) were sown in water-moistened sand in covered flats (10 rows, 5-6 seed/row) and covered with clear, plastic lids before being placed in a growth chamber having 16 hr light (25°C)/8 hr dark (20°C), 75% relative humidity and 13,000-14,000 LUX. One day after sowing, whole seeds were flash frozen in liquid nitrogen prior to storage at -80°C. Two days after sowing, embryos and endosperm were isolated and flash frozen in liquid nitrogen prior to storage at -80°C. On days 3-6, aerial tissues, roots and endosperm were isolated and flash frozen in liquid nitrogen prior to storage at -80°C.

(hh) Rough Sheath2-R (rs2-R) Mutants (1400-6/S-17)

This experiment was conducted to identify abnormally expressed genes in the shoot apex of *rough sheath2-R (rs2-R)* mutant plants. *rs2* encodes a myb domain DNA binding protein that functions in repression of several shoot apical meristem expressed homeobox genes. Two homeobox gene targets are known for *rs2* repression, *rough sheath1*, *liguleless 3*. The recessive loss of function phenotype of *rs2-R* homozygous plants is described in Schneeberger et al. 1998 *Development* 125: 2857-2865.

The seed stock genetically segregates 1:1 for *rs2-R/rs2-R : rs2-R/+*

Preparation of tissue samples: 160 seedlings pooled from 2 and 3 week old plants grown in sand. Growth conditions; Conviron #107 @ 12 hr days/12hr night, 25°C, 75% humidity. Shoot apex was dissected to include leaf three and older. (Pictures available upon request).

- 1) *rough sheath2-R* homozygous (mutant) shoot apex
- 2) *rough sheath2-R* heterozygous (wt, control) shoot apex

(ii) Leaf Mutant 3642:

Mutant 3642 is a recessive mutation that causes abnormal leaf development. The leaves of mutant 3642 plants are characterized by leaf twisting and irregular leaf shape. Mutant 3642 plants also exhibit abnormally shaped floral organs which results in reduced fertility.

Seed segregating for the mutant phenotype was sown in Metro-mix 350 soil and grown in a Conviron growth chamber with watering by sub-irrigation twice a week.

Environmental conditions were set at 20 degrees Celsius, 70% humidity with an 8 hour day, 16 hour night light regime. Plants were harvested after 4 weeks of growth and the entire aerial portion of the plant was harvested and immediately frozen in liquid nitrogen and stored at -80C. Mutant phenotype plants were harvested separately from normal phenotype plants, which serve as the control tissue.

(jj) Flowers (green, white or buds)

Approximately 10 µl of *Arabidopsis thaliana* seeds (ecotype Ws) were sown on 350 soil (containing 0.03% marathon) and vernalized at 4C for 3 days. Plants were then grown at room

temperature under fluorescent lighting until flowering. Flowers were harvested after 28 days in three different categories. Buds that had not opened at all and were completely green were categorized as "flower buds" (also referred to as green buds by the investigator). Buds that had started to open, with white petals emerging slightly were categorized as "green flowers" (also referred to as white buds by the investigator). Flowers that had opened mostly (with no siliques elongation) with white petals completely visible were categorized as "white flowers" (also referred to as open flowers by the investigator). Buds and flowers were harvested with forceps, flash frozen in liquid nitrogen and stored at -80C until RNA was isolated.

## 2. Microarray Hybridization Procedures

Microarray technology provides the ability to monitor mRNA transcript levels of thousands of genes in a single experiment. These experiments simultaneously hybridize two differentially labeled fluorescent cDNA pools to glass slides that have been previously spotted with cDNA clones of the same species. Each arrayed cDNA spot will have a corresponding ratio of fluorescence that represents the level of disparity between the respective mRNA species in the two sample pools. Thousands of polynucleotides can be spotted on one slide, and each experiment generates a global expression pattern.

### COATING SLIDES

The microarray consists of a chemically coated microscope slide, referred herein as a "chip" with numerous polynucleotide samples arrayed at a high density. The poly-L-lysine coating allows for this spotting at high density by providing a hydrophobic surface, reducing the spreading of spots of DNA solution arrayed on the slides. Glass microscope slides (Gold Seal #3010 manufactured by Gold Seal Products, Portsmouth, New Hampshire, USA) were coated with a 0.1%W/V solution of Poly-L-lysine (Sigma, St. Louis, Missouri) using the following protocol:

1. Slides were placed in slide racks (Shandon Lipshaw #121). The racks were then put in chambers (Shandon Lipshaw #121).

2. Cleaning solution was prepared:

70 g NaOH was dissolved in 280 mL ddH<sub>2</sub>O.

420 mL 95% ethanol was added. The total volume was 700 mL (= 2 X 350 mL); it was stirred until completely mixed.

If the solution remained cloudy, ddH<sub>2</sub>O was added until clear.

3. The solution was poured into chambers with slides; the chambers were covered with glass lids. The solution was mixed on an orbital shaker for 2 hr.

4. The racks were quickly transferred to fresh chambers filled with ddH<sub>2</sub>O. They were rinsed vigorously by plunging racks up and down.

Rinses were repeated 4X with fresh ddH<sub>2</sub>O each time, to remove all traces of NaOH-ethanol.

5. Polylysine solution was prepared:

0 mL poly-L-lysine + 70 mL tissue culture PBS in 560 mL water, using plastic graduated cylinder and beaker.

6. Slides were transferred to polylysine solution and shaken for 1 hr.

7. The rack was transferred to a fresh chambers filled with ddH<sub>2</sub>O. It was plunged up and down 5X to rinse.

8. The slides were centrifuged on microtiter plate carriers (paper towels were placed below the rack to absorb liquid) for 5 min. @ 500 rpm. The slide racks were transferred to empty chambers with covers.

9. Slide racks were dried in a 45C oven for 10 min.

10. The slides were stored in a closed plastic slide box.

11. Normally, the surface of lysine coated slides was not very hydrophobic immediately after this process, but became increasingly hydrophobic with storage. A hydrophobic surface helped ensure that spots didn't run together while printing at high densities. After they aged for 10 days to a month the slides were ready to use. However, coated slides that have been sitting around for long periods of time were usually too old to be used. This was because they developed opaque patches, visible when held to the light, and these resulted in high background hybridization from the fluorescent probe.

Alternativey, precoated glass slides were purchased from TeleChem Internation, Inc. (Sunnyvale, CA, 94089; catalog number SMM-25, Superamine substrates).

#### PCR AMPLIFICATION OF cDNA CLONE INSERTS

Polynucleotides were amplified from *Arabidopsis* cDNA clones using insert specific probes. The resulting 100uL PCR reactions were purified with Qiaquick 96 PCR purification columns (Qiagen, Valencia, California, USA) and eluted in 30 uL of 5mM Tris. 8.5uL of the elution were mixed with 1.5uL of 20X SSC to give a final spotting solution of DNA in 3X SSC. The concentrations of DNA generated from each clone varied between 10-100 ng/ul, but were usually about 50 ng/ul.

#### ARRAYING OF PCR PRODUCTS ON GLASS SLIDES

PCR products from cDNA clones were spotted onto the poly-L-Lysine coated glass slides using an arrangement of quill-tip pins (ChipMaker 3 spotting pins; Telechem, International, Inc., Sunnyvale, California, USA) and a robotic arrayer (PixSys 3500, Cartesian Technologies, Irvine, California, USA). Around 0.5 nl of a prepared PCR product was spotted at each location to produce spots with approximately 100um diameters. Spot center-to-center spacing was from 180 um to 210um depending on the array. Printing was conducted in a chamber with relative humidity set at 50%.

Slides containing maize sequences were purchased from Agilent Technology (Palo Alto, CA 94304).

POST-PROCESSING OF SLIDES

After arraying, slides were processed through a series of steps – rehydration, UV cross-linking, blocking and denaturation - required prior to hybridization. Slides were rehydrated by placing them over a beaker of warm water (DNA face down), for 2-3 sec, to distribute the DNA more evenly within the spots, and then snap dried on a hot plate (DNA side, face up). The DNA was then cross-linked to the slides by UV irradiation (60-65mJ; 2400 Stratalinker, Stratagene, La Jolla, California, USA).

Following this a blocking step was performed to modify remaining free lysine groups, and hence minimize their ability to bind labeled probe DNA. To achieve this the arrays were placed in a slide rack. An empty slide chamber was left ready on an orbital shaker. The rack was bent slightly inwards in the middle, to ensure the slides would not run into each other while shaking. The blocking solution was prepared as follows:

3x 350-ml glass chambers (with metal tops) were set to one side, and a large round Pyrex dish with dH<sub>2</sub>O was placed ready in the microwave. At this time, 15ml sodium borate was prepared in a 50 ml conical tube.

6-g succinic anhydride was dissolved in approx. 325-350 mL 1-methyl-2-pyrrolidinone. Rapid addition of reagent was crucial.

- a. Immediately after the last flake of the succinic anhydride dissolved, the 15-mL sodium borate was added.
- b. Immediately after the sodium borate solution mixed in, the solution was poured into an empty slide chamber.
- c. The slide rack was plunged rapidly and evenly in the solution. It was vigorously shaken up and down for a few seconds, making sure slides never left the solution.
- d. It was mixed on an orbital shaker for 15-20 min. Meanwhile, the water in the Pyrex dish (enough to cover slide rack) was heated to boiling.

Following this, the slide rack was gently plunge in the 95C water (just stopped boiling) for 2 min. Then the slide rack was plunged 5X in 95% ethanol. The slides and rack were centrifuged for 5 min. @ 500 rpm. The slides were loaded quickly and evenly onto the carriers to avoid streaking. The arrays were used immediately or store in slide box.

The Hybridization process began with the isolation of mRNA from the two tissues (see “*Isolation of total RNA*” and “*Isolation of mRNA*”, below) in question followed by their conversion to single stranded cDNA (see “*Generation of probes for hybridization*”, below). The cDNA from each tissue was independently labeled with a different fluorescent dye and then both samples were pooled together. This final differentially labeled cDNA pool was then placed on a processed microarray and allowed to hybridize (see “*Hybridization and wash conditions*”, below).

#### ISOLATION OF TOTAL RNA

Approximately 1 g of plant tissue was ground in liquid nitrogen to a fine powder and transferred into a 50-ml centrifuge tube containing 10 ml of Trizol reagent. The tube was vigorously vortexed for 1 min and then incubated at room temperature for 10-20 min. on an orbital shaker at 220 rpm. Two ml of chloroform was added to the tube and the solution vortexed vigorously for at least 30-sec before again incubating at room temperature with shaking. The sample was then centrifuged at 12,000 X g (10,000 rpm) for 15-20 min at 4°C. The aqueous layer was removed and mixed by inversion with 2.5 ml of 1.2 M NaCl/0.8 M Sodium Citrate and 2.5 ml of isopropyl alcohol added. After a 10 min. incubation at room temperature, the sample was centrifuged at 12,000 X g (10,000 rpm) for 15 min at 4°C. The pellet was washed with 70% ethanol, re-centrifuged at 8,000 rpm for 5 min and then air dried at room temperature for 10 min. The resulting total RNA was dissolved in either TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or DEPC (diethylpyrocarbonate) treated deionized water (RNase-free water). For subsequent isolation of mRNA using the Qiagen kit, the total RNA pellet was dissolved in RNase-free water.

#### ISOLATION OF mRNA

mRNA was isolated using the Qiagen Oligotex mRNA Spin-Column protocol (Qiagen, Valencia, California). Briefly, 500 µl OBB buffer (20 mM Tris-Cl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS) was added to 500 µl of total RNA (0.5 – 0.75 mg) and mixed thoroughly.

The sample was first incubated at 70°C for 3 min, then at room temperature for 10 minutes and finally centrifuged for 2 min at 14,000 – 18,000 X g. The pellet was resuspended in 400 µl OW2 buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA) by vortexing, the resulting solution placed on a small spin column in a 1.5 ml RNase-free microcentrifuge tube and centrifuged for 1 min at 14,000 – 18,000 X g. The spin column was transferred to a new 1.5 ml RNase-free microcentrifuge tube and washed with 400 µl of OW2 buffer. To release the isolated mRNA from the resin, the spin column was again transferred to a new RNase-free 1.5 ml microcentrifuge tube, 20-100 µl 70°C OEB buffer (5 mM Tris-Cl, pH 7.5) added and the resin resuspended in the resulting solution via pipeting. The mRNA solution was collected after centrifuging for 1 min at 14,000 – 18,000 X g.

Alternatively, mRNA was isolated using the Stratagene Poly(A) Quik mRNA Isolation Kit (Stratagene, La Jolla, California). Here, up to 0.5 mg of total RNA (maximum volume of 1 ml) was incubated at 65°C for 5 minutes, snap cooled on ice and 0.1X volumes of 10X sample buffer (10mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0) 5 M NaCl) added. The RNA sample was applied to a prepared push column and passed through the column at a rate of ~1 drop every 2 sec. The solution collected was reapplied to the column and collected as above. 200 µl of high salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 NaCl) was applied to the column and passed through the column at a rate of ~1 drop every 2 sec. This step was repeated and followed by three low salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl) washes preformed in a similar manner. mRNA was eluted by applying to the column four separate 200 µl aliquots of elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA) preheated to 65°C. Here, the elution buffer was passed through the column at a rate of 1 drop/sec. The resulting mRNA solution was precipitated by adding 0.1X volumes of 10X sample buffer, 2.5 volumes of ice-cold 100% ethanol, incubating overnight at -20°C and centrifuging at 14,000-18,000 X g for 20-30 min at 4°C. The pellet was washed with 70% ethanol and air dried for 10 min. at room temperature before resuspension in RNase-free deionized water.

#### PREPARATION OF YEAST CONTROLS

Plasmid DNA was isolated from the following yeast clones using Qiagen filtered maxiprep kits (Qiagen, Valencia, California): YAL022c(Fun26), YAL031c(Fun21), YBR032w,

YDL131w, YDL182w, YDL194w, YDL196w, YDR050c and YDR116c. Plasmid DNA was linearized with either *Bsr*BI (YAL022c(Fun26), YAL031c(Fun21), YDL131w, YDL182w, YDL194w, YDL196w, YDR050c) or *Afl*III (YBR032w, YDR116c) and isolated.

In Vitro Transcription of Yeast Clones

The following solution was incubated at 37°C for 2 hours: 17 µl of isolated yeast insert DNA (1 µg), 20 µl 5X buffer, 10 µl 100 mM DTT, 2.5 µl (100 U) RNasin, 20 µl 2.5 mM (ea.) rNTPs, 2.7 µl (40U) SP6 polymerase and 27.8 µl RNase-free deionized water. 2 µl (2 U) Ampli DNase I was added and the incubation continued for another 15 min. 10 µl 5M NH<sub>4</sub>OAC and 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) were added, the solution vortexed and then centrifuged to separate the phases. To precipitate the RNA, 250 µl ethanol was added and the solution incubated at -20°C for at least one hour. The sample was then centrifuged for 20 min at 4°C at 14,000-18,000 X g, the pellet washed with 500 µl of 70% ethanol, air dried at room temperature for 10 min and resuspended in 100 µl of RNase-free deionized water. The precipitation procedure was then repeated.

Alternatively, after the two-hour incubation, the solution was extracted with phenol/chloroform once before adding 0.1 volume 3M sodium acetate and 2.5 volumes of 100% ethanol. The solution was centrifuged at 15,000rpm, 4°C for 20 minutes and the pellet resuspended in RNase-free deionized water. The DNase I treatment was carried out at 37°C for 30 minutes using 2 U of Ampli DNase I in the following reaction condition: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>. The DNase I reaction was then stopped with the addition of NH<sub>4</sub>OAC and phenol:chloroform:isoamyl alcohol (25:24:1), and RNA isolated as described above.

0.15-2.5 ng of the *in vitro* transcript RNA from each yeast clone were added to each plant mRNA sample prior to labeling to serve as positive (internal) probe controls.

GENERATION OF PROBES FOR HYBRIDIZATION

Generation of labeled probes for hybridization from first-strand cDNA

Hybridization probes were generated from isolated mRNA using an Atlas™ Glass Fluorescent Labeling Kit (Clontech Laboratories, Inc., Palo Alto, California, USA). This entails a two step labeling procedure that first incorporates primary aliphatic amino groups during

cDNA synthesis and then couples fluorescent dye to the cDNA by reaction with the amino functional groups. Briefly, 5 µg of oligo(dT)<sub>18</sub> primer d(TTTTTTTTTTTTTTTTV) was mixed with Poly A+ mRNA (1.5 - 2 µg mRNA isolated using the Qiagen Oligotex mRNA Spin-Column protocol or the Stratagene Poly(A) Quik mRNA Isolation protocol (Stratagene, La Jolla, California, USA)) in a total volume of 25 µl. The sample was incubated in a thermocycler at 70°C for 5 min, cooled to 48°C and 10 µl of 5X cDNA Synthesis Buffer (kit supplied), 5 µl 10X dNTP mix (dATP, dCTP, dGTP, dTTP and aminoallyl-dUTP; kit supplied), 7.5 µl deionized water and 2.5 µl MMLV Reverse Transcriptase (500U) added. The reaction was then incubated at 48°C for 30 minutes, followed by 1hr incubation at 42°C. At the end of the incubation the reaction was heated to 70°C for 10 min, cooled to 37°C and 0.5 µl (5 U) RNase H added, before incubating for 15 min at 37°C. The solution was vortexed for 1 min after the addition of 0.5 µl 0.5 M EDTA and 5 µl of QuickClean Resin (kit supplied) then centrifuged at 14,000-18,000 X g for 1 min. After removing the supernatant to a 0.45 µm spin filter (kit supplied), the sample was again centrifuged at 14,000-18,000 X g for 1 min, and 5.5 µl 3 M sodium acetate and 137.5 µl of 100% ethanol added to the sample before incubating at -20°C for at least 1 hr. The sample was then centrifuged at 14,000-18,000 X g at 4°C for 20 min, the resulting pellet washed with 500 µl 70% ethanol, air-dried at room temperature for 10 min and resuspended in 10 µl of 2X fluorescent labeling buffer (kit provided). 10 µl each of the fluorescent dyes Cy3 and Cy5 (Amersham Pharmacia (Piscataway, New Jersey, USA); prepared according to Atlas™ kit directions of Clontech) were added and the sample incubated in the dark at room temperature for 30 min.

The fluorescently labeled first strand cDNA was precipitated by adding 2 µl 3M sodium acetate and 50 µl 100% ethanol, incubated at -20°C for at least 2 hrs, centrifuged at 14,000-18,000 X g for 20 min, washed with 70% ethanol, air-dried for 10 min and dissolved in 100 µl of water.

Alternatively, 3-4 µg mRNA, 2.5 (~8.9 ng of in vitro translated mRNA) µl yeast control and 3 µg oligo dTV (TTTTTTTTTTTTTT(A/C/G); Sequence ID No.: X) were mixed in a total volume of 24.7 µl. The sample was incubated in a thermocycler at 70°C for 10 min. before chilling on ice. To this, 8 µl of 5X first strand buffer (SuperScript II RNase H- Reverse

Transcriptase kit from Invitrogen (Carlsbad, California 92008); cat no. 18064022), 0.8 °C of aa-dUTP/dNTP mix (50X; 25mM dATP, 25mM dGTP, 25mM dCTP, 15mM dTTP, 10mM aminoallyl-dUTP), 4 µl of 0.1 M DTT and 2.5 µl (500 units) of Superscript R.T.II enzyme (Stratagene) were added. The sample was incubated at 42°C for 2 hours before a mixture of 10 °C of 1M NaOH and 10°C of 0.5 M EDTA were added. After a 15 minute incubation at 65°C, 25 µl of 1 M Tris pH 7.4 was added. This was mixed with 450 µl of water in a Microcon 30 column before centrifugation at 11,000 X g for 12 min. The column was washed twice with 450 µl (centrifugation at 11,000 g, 12 min.) before eluting the sample by inverting the Microcon column and centrifuging at 11,000 X g for 20 seconds. Sample was dehydrated by centrifugation under vacuum and stored at -20°C.

Each reaction pellet was dissolved in 9 µl of 0.1 M carbonate buffer (0.1M sodium carbonate and sodium bicarbonate, pH=8.5-9) and 4.5 µl of this placed in two microfuge tubes. 4.5 µl of each dye (in DMSO) were added and the mixture incubated in the dark for 1 hour. 4.5 µl of 4 M hydroxylamine was added and again incubated in the dark for 15 minutes.

Regardless of the method used for probe generation, the probe was purified using a Qiagen PCR cleanup kit (Qiagen, Valencia, California, USA), and eluted with 100 ul EB (kit provided). The sample was loaded on a Microcon YM-30 (Millipore, Bedford, Massachusetts, USA) spin column and concentrated to 4-5 ul in volume. Probes for the maize microarrays were generated using the Fluorescent Linear Amplification Kit (cat. No. G2556A) from Agilent Technologies (Palo Alto, CA).

#### HYBRIDIZATION AND WASH CONDITIONS

The following Hybridization and Washing Condition were developed:

#### Hybridization Conditions:

Labeled probe was heated at 95°C for 3 min and chilled on ice. Then 25 !L of the hybridization buffer which was warmed at 42C was added to the probe, mixing by pipetting, to give a final concentration of:

50% formamide  
4x SSC  
0.03% SDS  
5x Denhardt's solution  
0.1 µg/ml single-stranded salmon sperm DNA

The probe was kept at 42C. Prior to the hybridization, the probe was heated for 1 more min., added to the array, and then covered with a glass cover slip. Slides were placed in hybridization chambers (Telechem, Sunnyvale, California) and incubated at 42<sup>0</sup>C overnight.

Washing Conditions:

- A. Slides were washed in 1x SSC + 0.03% SDS solution at room temperature for 5 minutes,
- B. Slides were washed in 0.2x SSC at room temperature for 5 minutes,
- C. Slides were washed in 0.05x SSC at room temperature for 5 minutes.

After A, B, and C, slides were spun at 800 x g for 2 min. to dry. They were then scanned.

Maize microarrays were hybridized according to the instructions included Fluorescent Linear Amplification Kit (cat. No. G2556A) from Agilent Technologies (Palo Alto, CA).

SCANNING OF SLIDES

The chips were scanned using a ScanArray 3000 or 5000 (General Scanning, Watertown, Massachusetts, USA). The chips were scanned at 543 and 633nm, at 10 um resolution to measure the intensity of the two fluorescent dyes incorporated into the samples hybridized to the chips.

DATA EXTRACTION AND ANALYSIS

The images generated by scanning slides consisted of two 16-bit TIFF images representing the fluorescent emissions of the two samples at each arrayed spot. These images were then quantified and processed for expression analysis using the data extraction software

Imagene<sup>TM</sup> (Biodiscovery, Los Angeles, California, USA). Imagene output was subsequently analyzed using the analysis program Genespring<sup>TM</sup> (Silicon Genetics, San Carlos, California, USA). In Genespring, the data was imported using median pixel intensity measurements derived from Imagene output. Background subtraction, ratio calculation and normalization were all conducted in Genespring. Normalization was achieved by breaking the data into 32 groups, each of which represented one of the 32 pin printing regions on the microarray. Groups consist of 360 to 550 spots. Each group was independently normalized by setting the median of ratios to one and multiplying ratios by the appropriate factor.

The results of the microarray experiments are reported in the MA\_DIFF Table as described above in the section entitled "Brief Description of the Individual Tables".

#### **EXAMPLE 4: AFLP EXPERIMENTS AND RESULTS**

##### **Production of Samples**

mRNA was prepared from 27 plant tissues. Based on preliminary cDNA-AFLP analysis with a few primer combinations, 11 plant tissues and/or pooled samples were selected. Samples were selected to give the greatest representation of unique band upon electrophoresis. The final 11 samples or pooled samples used in the cDNA-AFLP analysis were:

- S1 Dark adapted seedlings
- S2 Roots/Etiolated Seedlings
- S3 Mature leaves, soil grown
- S4 Immature buds, inflorescence meristem
- S5 Flowers opened
- S6 Siliques, all stages
- S7 Senescing leaves (just beginning to yellow)
- S8 Callus Inducing medium
  - Callus shoot induction
  - Callus root induction

S9	Wounding
	Methyl-jasmonate-treated
S10	Oxidative stress
	Drought stress
	Oxygen Stress-flooding
S11	Heat treated light grown seedling
	Cold treated light grown seedlings

cDNA from each of the 11 samples was digested with two restriction endonucleases, namely *TaqI* and *MseI*. *TaqI* and *MseI* adapters were then ligated to the restriction enzyme fragments. Using primers to these adapters that were specific in sequence (i.e. without extensions), the restriction fragments were subjected to cycles of non-radioactive pre-amplification.

#### Selective PCR

In order to limit the number of fragments or bands on each lane of the AFLP gel, fragments were subjected to another round of selective radioactive polymerase chain amplification. The *TaqI* primers used in this amplification were 5'-labelled with P<sup>33</sup>. For these amplifications, the *TaqI* primers had two extra nucleotides at their 3' end and the *MseI* primers had three extra nucleotides at their 3' end. This resulted in 16 primer designs for the *TaqI* primer and 64 primer designs for the *MseI* primer. Altogether, this gave rise to a total of 1024 primer designs. Fragments generated in this selective amplification protocol were run with labeled molecular weight markers on polyacrylamide gels to separate fragments in the size range of 100 – 600 nucleotides.

Following gel electrophoresis, profiles were analyzed with a phosphoimager. From these images, electronic files, giving the mobilities of all bands on the gels and their intensities in each of the samples, were compiled.

All unique bands were cut out of the gels. The gel pieces were placed in 96 well plates for elution and their plate designation was linked to their electrophoretic mobilities recorded in the electronic files. The eluted fragments were then subjected to another round of amplification,

this time using reamplification primers (see below). After amplification, DNA fragments were sequenced.

A computer database was established linking the mobilities of all the bands observed on the cDNA-AFLP gels with the sequence of the correspondingly isolated fragment. The sequence allowed for identification of the gene from which the cDNA-AFLP fragment was derived, allowing for a linkage of band mobility with the transcript of a specific gene. Also linked to the band mobilities were their intensities recorded for each of the eleven samples used in constructing the database.

This cDNA-AFLP analysis with *TaqI/MseI* and 1024 primer combinations was repeated using the enzymes *NlaIII* in place of *TaqI*, and *Csp6I* in place of *MseI*.

Using the Database for the Transcript Profiling of Experimental Samples

Experimental Samples were subjected to cDNA-AFLP as described above, resulting in electronic files recording band mobilities and intensities. Through use of the database established above, band mobilities could be linked to specific cDNAs, and therefore genes. Furthermore, the linkage with the intensities in the respective samples allowed for the quantification of specific cDNAs in these samples, and thus the relative concentration of specific transcripts in the samples, indicating the level to which specific genes were expressed.

Reamplification primers

99G24

CGCCAGGGTTTCCCAGTCACGAC|ACGACTCACT|gatgagtccctgagtaa|

M13 forward                    +10                    MseI+0

99G20

AGCGGATAACAATTTCACACAGGA|CACACTGGTA|tagactgcgtaccga|

M13 reverse                    +10                    TaqI +0

Purification of the Reamplification reaction before sequencing

5 µl reamplification reaction  
0,25 µl 10xPCR buffer  
0,33 µl Shrimp Alkaline Phosphatase (Amersham Life Science)  
0,033 µl Exonuclease I (USB)  
0,297 µl SAP dilution buffer  
1,59 µl MQ  
7.5 µl total

30' 37°C

10' 80°C

4°C

Sample Preparation

S1: Dark adapted seedlings: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 8 days, the seedlings were foil-wrapped and harvested after two days.

S2: Roots/Etiolated seedlings: Seeds of *Arabidopsis thaliana* (wassilewskija) were germinated on solid germination media (1X MS salts, 1X MS vitamins, 20g/L sucrose, 50 mg/L MES pH 5.8) in the dark. Tissues were harvested 14 days later.

S3: Mature leaves, soil grown: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. Leaves were harvested 17 days later from plants that had not yet bolted.

**S4: Immature buds, inflorescence meristem:** Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark.

**S5: Flowers, opened:** Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark.

**S6: Siliques, all stages:** Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark.

**S7: Senescing leaves (just beginning to yellow):** Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. When the plant had leaves that were less than 50% yellow, the leaves that were just beginning to yellow were harvested.

S8:

**Callus Inducing Medium:** Seeds of *Arabidopsis thaliana* (wassilewskija) were surface sterilized (1 min-75% Ethanol, 6 min-bleach 100% + Tween 20, rinse) and incubated on MS medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) 1 mg/l and Kinetin 1 mg/l in the dark for 3 weeks to generate primary callus.

Hypocotyls and roots of the seedling were swollen after a week after incubation in this callus induction medium and subsequently callus was initiated from these swollen areas.

**Callus shoot induction:** Primary calluses were transferred to the fresh callus induction medium for another 2 weeks growth to generate secondary callus. Secondary callus were

transferred to shoot induction medium containing MS basal medium and Benzyladenine (BA) 2 mg/l and Naphthaleneacetic acid (NAA) .1 mg/l for 2 weeks growth in the light before it was harvested and frozen and sent to Keygene. Many shoot meristems were observed under the microscope.

Callus root induction: Secondary calluses were transferred to root induction medium containing MS basal medium, sucrose 1% and Indolebutyric acid (IBA) 0.05 mg/l in the dark. Many root primordia were observed under microscope after 10 days in the root induction medium. Those callus tissue were harvested and frozen and sent to Keygene.

S9:

Wounding: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 20 days, leaves of plants were wounded with pliers. Wounded leaves were harvested 1 hour and 4 hours after wounding.

Methyl jasmonate treatment: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 13 days, plants were sprayed with 0.001% methyl jasmonate. Leaves were harvested 1.5 hours and 6 hours after spraying

S10:

Oxidative stress: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 24 days, a few leaves were inoculated with a mixture of 2.5 mM D-glucose, 2.5 U/mL glucose oxidase in 20 mM sodium phosphate buffer pH 6.5. After an hour, 3 hours, or 5 hours after inoculation, whole plant, except for the inoculated leaves, was

harvested. This sample was mixed with sample from plants that were sitting in full sun (152,000 LUX) for 2 hours or four hours.

Drought stress: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 20 days, aerial tissues were harvested and left to dry in 3MM Whatman paper for 1 hour or 4 hours.

Oxygen stress: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 21 days, the plant was flooded by immersing its pot in a beaker of tap water. After 6 days, the upper tissues were harvested.

S11: Heat-treated light grown seedlings: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. Over a 5 hour period, the temperature was raised to 42°C at the rate of approximately 4°C per hour. After 1 hour at 42°C, the aerial tissues were collected. This sample was mixed with an equal volume of sample that went through a heat-recovery treatment namely bringing down the temperature to 22°C from 42°C over a 5 hour period at the rate of 4°C per hour.

Cold-treated light grown seedlings: Seeds of *Arabidopsis thaliana* (wassilewskija) were sown in pots and left at 4°C for two to three days to vernalize. They were transferred to a growth chamber after three days. The intensity of light in the growth chamber was 7000-8000 LUX, temperature was 22°C, with 16 h light and 8 h dark. After 18 days, the plant was transferred to

4°C for an hour before the aerial tissues were harvested. This sample was mixed with aerial tissues from another plant that was transferred to 4°C for 27 hours before being harvested.

Analysis of Data:

Intensity: The intensity of the band corresponds to the value in each lane marked S1, S2 etc.

P-values: The data shows P- values of each of the samples 1-11. P-values are calculated using the following formula  $2*(1-NORMDIST(ABS(Sx-AVERAGE(S1 to S11, not including Sx))/STDEV(S1 to S11 not including Sx),0,1,TRUE))$  using Excel functions.

The equivalent mathematical formula of P-value is as follows:

$\int \phi(x) dx$ , integrated from a to  $\infty$ ,

where  $\phi(x)$  is a normal distribution:

where  $a = |Sx - \mu|$

$\sigma(S1 \dots S11, \text{not including } Sx);$

where  $\mu$  = is the average of the intensities of all samples except  $Sx$ ,

$$= \frac{(\sum S1 \dots Sn) - Sx}{n-1}$$

where  $\sigma(S1 \dots S11, \text{not including } Sx)$  = the standard deviation of all sample intensities except  $Sx$ .

Results:

The results are shown in the MA\_diff tables.

**EXAMPLE 5: TRANSFORMATION OF CARROT CELLS**

Transformation of plant cells can be accomplished by a number of methods, as described above. Similarly, a number of plant genera can be regenerated from tissue culture following transformation. Transformation and regeneration of carrot cells as described herein is illustrative.

Single cell suspension cultures of carrot (*Daucus carota*) cells are established from hypocotyls of cultivar Early Nantes in B<sub>5</sub> growth medium (O.L. Gamborg et al., *Plant Physiol.* 45:372 (1970)) plus 2,4-D and 15 mM CaCl<sub>2</sub> (B<sub>5</sub>-44 medium) by methods known in the art. The suspension cultures are subcultured by adding 10 ml of the suspension culture to 40 ml of B<sub>5</sub>-44 medium in 250 ml flasks every 7 days and are maintained in a shaker at 150 rpm at 27 °C in the dark.

The suspension culture cells are transformed with exogenous DNA as described by Z. Chen et al. *Plant Mol. Bio.* 36:163 (1998). Briefly, 4-days post-subculture cells are incubated with cell wall digestion solution containing 0.4 M sorbitol, 2% driselase, 5mM MES (2-[N-Morpholino] ethanesulfonic acid) pH 5.0 for 5 hours. The digested cells are pelleted gently at 60 xg for 5 min. and washed twice in W5 solution containing 154 mM NaCl, 5 mM KCl, 125 mM CaCl<sub>2</sub> and 5mM glucose, pH 6.0. The protoplasts are suspended in MC solution containing 5 mM MES, 20 mM CaCl<sub>2</sub>, 0.5 M mannitol, pH 5.7 and the protoplast density is adjusted to about 4 x 10<sup>6</sup> protoplasts per ml.

15-60 µg of plasmid DNA is mixed with 0.9 ml of protoplasts. The resulting suspension is mixed with 40% polyethylene glycol (MW 8000, PEG 8000), by gentle inversion a few times at room temperature for 5 to 25 min. Protoplast culture medium known in the art is added into the PEG-DNA-protoplast mixture. Protoplasts are incubated in the culture medium for 24 hour to 5 days and cell extracts can be used for assay of transient expression of the introduced gene. Alternatively, transformed cells can be used to produce transgenic callus, which in turn can be used to produce transgenic plants, by methods known in the art. See, for example, Nomura and Komamine, *Plt. Phys.* 79:988-991 (1985), *Identification and Isolation of Single Cells that Produce Somatic Embryos in Carrot Suspension Cultures*.

#### **EXAMPLE 6: PHENOTYPE SCREENS AND RESULTS**

##### **A: Triparental Mating and Vacuum Infiltration Transformation of Plants**

Standard laboratory techniques are as described in Sambrook et al. (1989) unless otherwise stated. Single colonies of *Agrobacterium* C58C1Rif, *E. coli* helper strain HB101 and the *E. coli* strain containing the transformation construct to be mobilized into *Agrobacterium*

were separately inoculated into appropriate growth media and stationary cultures produced. 100 µl of each of the three cultures were mixed gently, plated on YEB (5g Gibco beef extract, 1g Bacto yeast extract, 1g Bacto peptone, 5g sucrose, pH 7.4) solid growth media and incubated overnight at 28°C. The bacteria from the triparental mating were collected in 2 ml of lambda buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>) and serial dilutions made. An aliquot of the each dilution was then plated and incubated for 2 days at 28°C on YEB plates supplemented with 100 µg/ml rifampicin and 100 µg/ml carbenicillin for calculation of the number of acceptor cells and on YEB plates supplemented with 100 µg/ml rifampicin, 100 µg/ml carbenicillin and 100 µg/ml spectinomycin for selection of transconjugant cells. The cointegrate structure of purified transconjugants was verified via Southern blot hybridization.

A transconjugant culture was prepared for vacuum infiltration by inoculating 1 ml of a stationary culture arising from a single colony into liquid YEB media and incubating at 28°C for approximately 20 hours with shaking (220 rpm) until the OD taken at 600 nm was 0.8-1.0. The culture was then pelleted (8000 rpm, 10 min, 4°C in a Sorvall SLA 3000 rotor) and the bacteria resuspended in infiltration medium (0.5X MS salts, 5% w/v sucrose, 10 µg/l BAP, 200 µl/l Silwet L-77, pH 5.8) to a final OD<sub>600</sub> of 1.0. This prepared transconjugant culture was used within 20 minutes of preparation.

Wild-type plants for vacuum infiltration were grown in 4-inch pots containing Metromix 200 and Osmocote. Briefly, seeds of *Arabidopsis thaliana* (ecotype Wassilewskija) were sown in pots and left at 4°C for two to four days to vernalize. They were then transferred to 22-25°C and grown under long-day (16 hr light: 8 hr dark) conditions, sub-irrigated with water. After bolting, the primary inflorescence was removed and, after four to eight days, the pots containing the plants were inverted in the vacuum chamber to submerge all of the plants in the prepared transconjugant culture. Vacuum was drawn for two minutes before pots were removed, covered with plastic wrap and incubated in a cool room under darkness or very low light for one to two days. The plastic wrap was then removed, the plants returned to their previous growing conditions and subsequently produced (T1) seed collected.

B: Selection of T-DNA Insertion Lines

Approximately 10,750 seeds from the initial vacuum infiltrated plants were sown per flat of Metromix 350 soil. Flats were vernalized for four to five days at 4°C before being transferred to

22-25°C and grown under long-day (16 hr light: 8 hr dark) conditions, sub-irrigated with water. Approximately seven to ten days after germination, the (T1) seedlings were sprayed with 0.02% Finale herbicide (AgrEvo). After another five to seven days, herbicide treatment was repeated. Herbicide resistant T1 plants were allowed to self-pollinate and T2 seed were collected from each individual. In the few cases where the T1 plant produced few seed, the T2 seed was planted in bulk, the T2 plants allowed to self-pollinate and T3 seed collected.

### C: Phenotype Screening

Approximately 40 seed from each T2 (or T3) line were planted in a 4-inch pot containing either Sunshine mix or Metromix 350 soil. Pots were vernalized for four to five days at 4°C before being transferred to 22-25°C and grown under long-day (16 hr light: 8 hr dark) conditions, sub-irrigated with water. A first phenotype screen was conducted by visually inspecting the seedlings five to seven days after germination and aberrant phenotypes noted. Plants were then sprayed with Finale herbicide within four days (i.e. about seven to nine days after germination). The second visual screen was conducted on surviving T2 (or T3) plants about sixteen to seventeen days after germination and the final screen was conducted after the plants had bolted and formed siliques. Here, the third and fourth green siliques were collected and aberrant phenotypes noted. The Knock-in and Knock-out Tables contain descriptions of identified phenotypes.

Alternative, seed were surface sterilized and transferred to agar solidified medium containing Murashige and Skoog salts (1X), 1% sucrose (wt/v) pH 5.7 before autoclaving. Seed were cold treated for 48 hours and transferred to long days [16 hours light and 8 hours dark], 25°C. Plants were screened at 5 and 10 days.

In another screen, seed were surface sterilized and transferred to agar solidified medium containing Murashige and Skoog salts (1X), and combinations of various nitrogen and sucrose amounts as specified below:

Medium 1: no sucrose, 20.6 mM NH<sub>4</sub>NO<sub>3</sub>, 18.8 mM KNO<sub>3</sub>;  
Medium 2: 0.5% sucrose, 20.6 mM NH<sub>4</sub>NO<sub>3</sub>, 18.8 mM KNO<sub>3</sub>;  
Medium 3: 3% sucrose, 20.6 mM NH<sub>4</sub>NO<sub>3</sub>, 18.8 mM KNO<sub>3</sub>;  
Medium 4: no sucrose, 20.6 μM NH<sub>4</sub>NO<sub>3</sub>, 18.8 μM KNO<sub>3</sub>;  
Medium 5: 0.5% sucrose, 20.6 μM NH<sub>4</sub>NO<sub>3</sub>, 18.8 μM KNO<sub>3</sub>; and  
Medium 6: 3% sucrose, 20.6 μM NH<sub>4</sub>NO<sub>3</sub>, 18.8 μM KNO<sub>3</sub>.

The 0.5% sucrose was the control concentration for the sucrose. The low nitrogen, 20.6 μM NH<sub>4</sub>NO<sub>3</sub>, 18.8 μM KNO<sub>3</sub>, is the control for the nitrogen. Seeds were cold treated for 48 hours and transferred to long days [16 hours light and 8 hours dark], 25°C. Plants were screened at 2, 5, and 10 days.

#### D: TAIL-PCR and Fragment Sequencing

Rosette leaves were collected from each putative mutant and crushed between parafilm and FTA paper (Life Technologies). Two 2mm<sup>2</sup> hole punches were isolated from each FTA sample and washed according to the manufacturer's instructions by vortexing with 200 ul of the provided FTA purification reagent. The FTA reagent was removed and the washing procedure repeated two more times. The sample was then washed twice with 200 ul of FTA TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) and vortexing prior to PCR.

Primers used for TAIL-PCR are as follows:

AD2: 5' NGTCGASWGANA WGAA 3' (128-fold degeneracy)

S = G or C, W = A or T, and N = A, G, C, or T

LB1: 5' GTTTAACTGCGGCTCAACTGTCT 3'

LB2: 5' CCCATAGACCCCTACCGCTTAGTT 3'

LB3: 5' GAAAGAAAAAGAGGTATAACTGGTA 3'

The extent to which the left and right borders of the T-DNA insert were intact was measured for each line by PCR. The following components were mixed for PCR: 1 2mm<sup>2</sup> FTA sample, 38.75 µl distilled water, 5 µl 10X Platinum PCR buffer (Life Technologies), 2 µl 50 mM MgCl<sub>2</sub>, 1 µl 10 mM dNTPs, 1 µl 10 µM primer LB1 (or RB1 for analysis of the right border), 1 µl 10 µM primer LB3R (or RB3R for analysis of the right border) and 1.25 U Platinum Taq (Life Technologies). Cycling conditions were: 94°C, 10 sec.; thirty cycles of 94°C, 1 sec. - 54°C, 1 sec. - 72°C, 1 sec.; 72°C, 4 sec. The expected band size for an intact left border is bp, while an intact right border generates a bp band.

Fragments containing left or right border T-DNA sequence and adjacent genomic DNA sequence were obtained via PCR. First product PCR reactions use the following reaction mixture: 1 2mm<sup>2</sup> FTA sample, 12.44 µl distilled water, 2 µl 10X Platinum PCR buffer (Life Technologies), 0.6 µl 50 mM MgCl<sub>2</sub>, 0.4 µl 10 mM dNTPs, 0.4 µl 10 µM primer LB1 (or RB1 for analysis of the right border), 3 µl 20 µM primer AD2 and 0.8 U Platinum Taq (Life Technologies). Cycling conditions for these reactions were: 93°C, 1 min.; 95°C, 1 min.; three cycles of 94°C, 45 sec. - 62°C, 1 min. - 72°C, 2.5 min.; 94°C, 45 sec.; 25°C, 3 min.; ramp to 72°C in 3 min.; 72°C, 2.5 min.; fourteen cycles of 94°C, 20 sec. - 68°C, 1 min. - 72°C, 2.5 min. - 94°C, 20 sec.; - 68°C, 1 min. - 72°C, 2.5 min. - 94°C, 20 sec. - 44°C, 1 min. - 72°C, 2.5 min.; 72°C, 5 min.; end; ~4.5 hrs. For second product PCR reactions 1 µl of a 1:50 dilution of the first PCR product reaction was mixed with 13.44 µl distilled water, 2 µl 10X Platinum PCR buffer (Life Technologies), 0.6 µl 50 mM MgCl<sub>2</sub>, 0.4 µl 10 mM dNTPs, 0.4 µl 10 µM primer LB2 (or RB2 for analysis of the right border), 2 µl 20 µM primer AD2 and 0.8 U Platinum Taq (Life Technologies). Second product cycling conditions were: eleven cycles of 94°C, 20 sec. - 64°C, 1 min. - 72°C, 2.5 min. - 94°C, 20 sec. - 64°C, 1 min. - 72°C, 2.5 min. - 94°C, 20 sec. - 44°C, 1 min.; 72°C, 5 min.; end; ~3 hrs. Third product PCR reactions were prepared by first diluting 2 µl of the second PCR product with 98 µl of distilled water and then adding 1 µl of the dilution to 13.44 µl distilled water, 2 µl 10X Platinum PCR buffer (Life Technologies), 0.6 µl 50 mM MgCl<sub>2</sub>, 0.4 µl 10 mM dNTPs, 0.4 µl 10 µM primer LB3 (or RB3 for analysis of the right border), 2 µl 20 µM primer AD2 and 0.8 U Platinum Taq (Life Technologies). Third product cycling conditions were: twenty cycles of 94°C, 38 sec. - 44°C, 1 min. - 72°C, 2.5 min.; 72°C, 5 min.;

end; ~2 hrs. Aliquots of the first, second and third PCR products were electrophoresed on 1% TAE (40 mM Tris-acetate, 1 mM EDTA) to determine their size.

Reactions were purified prior to sequencing by conducting a final PCR reaction. Here, 0.25 µl Platinum PCR Buffer (Life Technologies), 0.1 µl 50 mM MgCl<sub>2</sub>, 3.3 U SAP shrimp alkaline phosphatase, 0.33 U Exonuclease and 1.781 µl distilled water were added to a 5 µl third product and the reaction cycled at 37°C, 30 min.; 80°C, 10 min.; 4°C indefinitely.

Di-deoxy "Big Dye" sequencing was conducted on Perkin-Elmer 3700 or 377 machines.

### KNOCK-IN EXPERIMENTS

For the following examples, a two-component system was constructed in a plant to ectopically express the desired cDNA.

First, a plant was generated by inserting a sequence encoding a transcriptional activator downstream of a desired promoter, thereby creating a first component where the desired promoter facilitates expression of the activator generated a plant. The first component also is referred to as the activator line.

Next, the second component is constructed by linking a desired cDNA to a sequence that the transcriptional activator can bind to and facilitate expression of the desired cDNA. The second component can be inserted into the activator line by transformation. Alternatively, the second component can be inserted into a separate plant, also referred to as the target line. Then, the target and activator lines can be crossed to generate progeny that have both components.

Two component lines were generated by both means.

#### Part I - From crosses

Target lines containing cDNA constructs are generated using the Agrobacterium-mediated transformation. Selected target lines are genetically crossed to activation lines (or promoter

lines). Generally, the promoter lines used are as described above. Evaluation of phenotypes is done on the resulting F1 progenies.

#### Part II - From Type I Supertransformation

Promoter activation lines (generally Vascular/Ovule/Young Seed/Embryo line, Seed/Epidermis/Ovary/Fruit line, Roots/Shoots/Ovule line, and Vasculature/Meristem are transformed with cDNA constructs using the Agrobacterium mediated transformation. Selected transformants (and their progenies) are evaluated for changes in phenotypes. The table for the knock-in of the Type I supertransformation comprises the following information

- Clone ID,
- Pfam,
- Gemini ID
- Trans. Unique ID (which indicates what promoter activation line was transformed)
- S Ratio: segregation ratio after the transformed plants are selected for the marker.
- Assay
- Stage: phenotype was observed
- Feature: Where the phenotype was observed
- Phenotype
- P Ratio: phenotype ratio
- Comments

#### Part III - From Type II Supertransformation

Target lines generated using the procedure mentioned in Part I are transformed with T-DNA construct containing constitutive promoter. Selected transformants (and their progenies) are evaluated for changes in phenotypes.

An additional deposit of an *E. coli* Library, *E. coli*LibA021800, was made at the American Type Culture Collection in Manassas, Virginia, USA on February 22, 2000 to meet the requirements of Budapest Treaty for the international recognition of the deposit of microorganisms. This deposit was assigned ATCC accession no. PTA-1411.

Additionaly, ATCC Library deposits; PTA-1161, PTA-1411 and PTA-2007 were made at the American Type Culture Collection in Manassas, Virginia, USA on; January 7, 2000, February 23, 2000 and June 8, 2000 respectively, to meet the requirements of Budapest Treaty for the international recognition of the deposit of microorganisms.

The invention being thus described, it will be apparent to one of ordinary skill in the art that various modifications of the materials and methods for practicing the invention can be made. Such modifications are to be considered within the scope of the invention as defined by the following claims.

Each of the references from the patent and periodical literature cited herein is hereby expressly incorporated in its entirety by such citation.